ASPECTS OF PURINE AND PYRIMIDINE METABOLISM

by

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This thesis is dedicated to the memory of my mother and father, Bunty and Colin; my wife Marguerite; my delightful daughter Julia; my faithful bull terrier Emma; and to all those animals who have, and continue to suffer in the name of science.

ABSTRACT

In Chapter 1 a review of the literature concerning aspects of erythrocyte membrane transport and metabolism, and purine and pyrimidine metabolism is presented.

The effects of pH, pO_2 and inorganic phosphate (Pi) on the uptake and metabolism of hypoxanthine by erythrocytes has been studied in Chapter 2. Uptake of hypoxanthine and accumulation of inosine 5'-monophosphate (IMP) were markedly increased at acid pH, high external phosphate concentrations, and low pO_2. Release of accumulated IMP as hypoxanthine occurred at alkaline pH values and low external phosphate concentrations. Conditions favouring IMP accumulation gave rise, in the absence of hypoxanthine, to a corresponding increase in 5'-phosphoribosyl-1-pyrophosphate (PRPP). Intracellular phosphate concentrations were markedly pH dependent and a model is presented whereby hypoxanthine uptake and release are controlled by intracellular concentrations of inorganic phosphate and 2,3-bisphosphoglycerate (2,3-DPG). These allosteric effectors influence, in opposing ways, two enzymes governing IMP accumulation, namely PRPP synthetase and 5'-nucleotidase. These metabolic properties suggest that the erythrocyte could play a role in the removal of hypoxanthine from anoxic tissue.

In Chapter 3 the kinetics and mechanism of transport of orotate across the human erythrocyte membrane and the effect of pH and inorganic phosphate on its metabolism (in the erythrocyte) have been studied. It has been shown that orotate enters erythrocytes with non-saturable kinetics and with a capacity of 190 μmoles/l packed cells/min at a concentration of 4-6 mmolar. The presence of competition for transport by a number of anions and the lack of competition by uridine is indicative of transport by a general anion transporter, with the ability for concentrative uptake in the absence of other external anions being compatible with transport via a ping-pong mechanism. Inhibition of transport by the specific band 3 inhibitors DIDS and CHCA confirm that transport is via the band 3 anion transporter. This explains the lack of significant uptake of orotate by most differentiated tissues which lack the intact band 3 protein. However, the demonstration of band 3 in rat hepatocytes (Cheng and Levy, 1980) provides a mechanism for the orotate transport which has been observed in liver (Handschumacher and Coleridge, 1979).

Changes in pH and inorganic phosphate (Pi) concentrations have been shown to have marked effects on the relative quantities of metabolic products produced by the erythrocyte from orotate. There was an increase in orotate metabolised with increasing Pi, an effect augmented by lowering the pH, and most easily explained by the allosteric activation of PRPP synthetase by Pi. The increase in UTP levels with

decreasing pH may be the consequence of both increased PRPP availability for the formation of uridine nucleotide from orotate, and decreased conversion of UMP to uridine by pyrimidine 5'-nucleotidase, which is known to be inhibited by phosphate. The accumulation of UDP sugars is optimal at a phosphate concentration of 10 mmolar, which is unexplained but would be compatible with an inhibitory effect of Pi on CTP synthetase.

A PRPP wasting cycle at alkaline pH values is proposed to explain the apparent paradox where no PRPP was observed to accumulate in erythrocytes (Chapter 2) at pH values of 7.6 and above in the presence of 10 mmolar phosphate and no added hypoxanthine, yet the metabolism of orotate, which is a PRPP utilising reaction, at alkaline pH values was readily demonstrable here. This (apparent paradox) can be resolved if one assumes that even in the absence of added hypoxanthine and demonstrable intracellular IMP there are sufficient quantities of hypoxanthine and/or IMP to maintain a PRPP wasting cycle at alkaline pH values. The cycle is interrupted at acidic pH values as phosphate levels rise and inhibit 5'-nucleotidase, an effect augmented by the decreasing levels of 2,3-DPG which accompany decreasing pH. This wasting cycle has recently been confirmed by P. Berman (unpublished).

The kinetics of orotate uptake by erythrocytes and its eventual release as uridine provides a role for the

erythrocyte in the transport and distribution of pyrimidines to peripheral tissues. A model is proposed and involves the de novo production of orotate in the liver. In the next step erythrocytes take up the orotate secreted by the liver into the circulation, convert it into an intermediate buffer store of uridine nucleotides, whose distribution is a function of pH and phosphate concentration, and eventually release it as uridine, which is a readily available form of pyrimidine for utilisation by peripheral nucleated cells. The enhancement of uptake of labelled orotate into nucleic acids of cultured cells is demonstrated here. The degradative half of the cycle proposes that uracil and β-alanine are the predominant degradative forms of pyrimidines produced by peripheral cells, and their ultimate metabolic fate is complete catabolism in the liver to CO_2 and water.

In the final chapter the possible role of the human erythrocyte in the prevention of reperfusion injury has been investigated. The development of a model of renal ischaemia in the rat is described. The ability of human erythrocytes, "primed" by preincubating in acid medium of high Pi concentration and low pO_2, to take up hypoxanthine in a concentrative manner when perfused through ischaemic rat kidney is demonstrated. Attempts to demonstrate improved survival and renal function in rats with "primed" human erythrocytes prior to reperfusion were, however, unsuccessful.

It is further demonstrated that "unprimed" human erythrocytes, resident in ischaemic rat kidney for 3 hours, take up hypoxanthine and convert it to IMP. This suggests that erythrocytes could play a physiological role in the prevention of reperfusion injury.

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The following publications have arisen out of the work undertaken for this thesis:

1. Harley, E.H., Sacks, S., Berman, P., Cohen, L., Simmonds, H.A., Fairbanks, L.D. and Black, D. Source and fate of circulating pyrimidines. Advances in Experimental Medicine and Biology 1986; 195A: 109-113.
2. Harley, E.H., Black, D., Berman, P. and Simmonds, H.A. Pyrimidine metabolism in the erythrocyte. Klinische Wocheschrift 1987; 65(Suppl. X): 22.
3. Berman, P.A., Black, D.A., Human, L. and Harley, E.H. An oxypurine cycle in human erythrocytes regulated by pH, inorganic phosphate and molecular oxygen. Klinische Wochenschrift 1987; 65(Suppl. X): 28.
4. Black, D.A., Harley, E.H. and Berman, P.A. Orotate uptake and metabolism by human erythrocytes. Klinische Wochenschrift 1987; 65(Suppl. X): 46.
5. Berman, P.A., Black, D.A., Human, L. and Harley, E.H. Oxypurine cycle in human erythrocytes regulated by pH, inorganic phosphate, and oxygen. Journal of Clinical Investigation 1988; 82: 980-986.

The following paper has been accepted for publication in the Journal of Chemical Education, subject to some minor changes: selective ion transport across biological membranes: a simple experiment in bioinorganic chemistry. Adams, P., and Black, D.A.

The following manuscript has recently been submitted to the Journal of Clinical Investigation for publication: Transport and metabolism of orotate by the human erythrocyte. Black, D.A., and Harley, E.H.

ABBREVIATIONS

ADA : adenosine deaminase
ADP : adenosine 5'-diphosphate
Ald : fructoaldolase
AMP : adenosine 5'-monophosphate
APRT : adenosine phosphoribosyl transferase
ATP : adenosine 5'-triphosphate
CHCA : alpha-cyano-4-hydroxycinnamate
Ci : Curie (2.2×10^{12} dpm)
cpm : counts per minute
DHAP : dihydroxyacetone phosphate
DIDS : 4,4-diisothiocyanostilbene-2,2'-disulfonate
DMEM : Dulbeccos minimal essential medium
DNA : deoxyribonucleic acid
1,3-DPG : 1,3-bisphosphoglycerate
2,3-DPG : 2,3-bisphosphoglycerate
DPGM : bisphosphoglycerate mutase
DPGP : 2,3-bisphosphoglycerate phosphatase
dpm : disintegrations per minute
E-4-P : erythrose-4-phosphate
EMP : Embden-Meyerhof pathway
En : enolase
FAD : flavine adenine dinucleotide (oxidised)
FCS : foetal calf serum
FDP : fructose-1,6-bisphosphatase
F-1,6-P : fructose-1,6-bisphosphate
F-6-P : fructose-6-phosphate
g : acceleration due to gravity
GDP : guanosine 5'-diphosphate
G-1,6-DP: glucose-1,6-bisphosphate
G-1-P : glucose-1-phosphate
G-6-P : glucose-6-phosphate
GA-3-P : glyceraldehyde-3-phosphate
GAPD : glyceraldehyde-3-phosphate dehydrogenase
GalK : galactokinase
Gal-1-P : galactose-1-phosphate
Gal-1-PUT: galactose-1-phosphate uridyl transferase
GSH : glutathione (reduced)

GSSH : glutathione (oxidised)
GTP : guanosine 5'-triphosphate
h : hour
Hb : haemoglobin
HEPES : 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid
HGPRT : hypoxanthine-guanine phosphoribosyl transferase
HK : hexokinase
HPLC : high pressure liquid chromatography
HPRT : hypoxanthine-guanine phosphoribosyltransferase
Hx : hypoxanthine
IDP : inosine 5'-diphosphate
IMP : inosine 5'-monophosphate
In : inosine
ITP : inosine 5'-triphosphate
kDa : kilodalton
Ki : inhibition constant
Km : Michaelis constant
L : litre
LDH : lactate dehydrogenase
LE : Long Evans
μ : micro
mg : milligram
min : minute
ml : millilitre
mmolar : millimolar
mM : millimolar
NAD : nicotinamide adenine dinucleotide (oxidised)
NADH : nicotinamide adenine dinucleotide (reduced)
NADP : nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH : nicotinamide adenine dinucleotide phosphate (reduced)
NAP-taurine: N-(4-azido-2-nitrophenyl)-2-aminoethyl-sulfonate
5'NT : purine 5'-nucleotidase
$O_2^{\bar{}}\cdot$: superoxide
OA : orotate
OH· : hydroxyl radical

OMP : orotate 5'-monophosphate
PCA : perchloric acid
PEP : phosphoenol pyruvate
PFK : phosphofructokinase
2-PG : 2-phosphoglycerate
3-PG : 3-phosphoglycerate
6-PG : 6-phosphogluconate
PGA : phosphoglycerate
PGI : phosphohexose isomerase
PGK : phosphoglycerate kinase
6-PGL : 6-phosphogluconolactone
PGM : phosphoglycerate mutase (phosphoglucomutase)
6-PGM : 6-phosphogluconate
6-PGnD : 6-phosphogluconate dehydrogenase
Pi : inorganic phosphate
PK : pyruvate kinase (purine kinase in Fig. 1.3)
PNP : purine nucleoside phosphorylase
PRPP : 5'-phosphoribosyl α-1-pyrophosphate
RBC : erythrocyte
RNA : ribonucleic acid
R-5-P : ribose-5-phosphate
Ru-5-P : ribulose-5-phosphate
SDS : sodium dodecyl sulphate
SOD : superoxide dismutase
S-7-P : sedoheptalose-7-phosphate
TDP : deoxythymidine 5'-diphosphate
TK : transketolase
TPI : triosephosphate isomerase
UDP : uridine 5'-diphosphate
UDPG : uridine 5'-diphosphate glucose
UDPGal : uridine 5'-diphosphate galactose
UDPGE : uridine 5'-diphosphate glucose epimerase
UMP : uridine 5'-monophosphate
UTP : uridine 5'-triphosphate
XD : xanthine dehydrogenase
XO : xanthine oxidase
X-P : phosphorylated glycolytic intermediates
X-5-P : xylulose-5-phosphate

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CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCTION

This thesis is to a large extent concerned with studies using the human erythrocyte. It would seem, therefore, that the brief review of general erythrocyte metabolism which follows is appropriate. Furthermore, more detailed accounts of specific areas dealt with in this thesis are given. These include the subjects of anion transport, purine transport and metabolism, and the physiology of 2,3-bisphosphoglycerate (2,3-DPG).

1.2 ERYTHROCYTE MATURATION

The human erythrocyte as ordinarily released into the circulation has lost its nucleus and associated DNA, and is therefore not capable of RNA synthesis. The residual messenger RNA and associated ribosomes, after loss of the nucleus, persist briefly (2 or 3 days) during which stage the cell is called a reticulocyte and is capable of some protein synthesis. It also briefly retains some mitochondria during this period. For the remainder of its life span of some 120 days the cell is incapable of protein synthesis, and must function with the proteins it has previously synthesised. Reticulocytes, unlike mature erythrocytes, are also able to synthesize lipids, haem and purines, and have an active

tricarboxylic acid (TCA) cycle and oxidative phosphorylation system.

After the reticulocyte stage, the cell loses its oxidative phosphorylation system. Oxygen consumption and carbon dioxide production by the mature red cell are much reduced and occur only through the pentose phosphate shunt pathway. Only remnants of the TCA cycle remain in the form of certain enzymes, and the cycle is inoperable as a system. The mature erythrocyte has lost most or all of its capabilities for lipid synthesis as well. The mature cell is also incapable of *de novo* purine sythesis. Purine metabolism will be considered in greater detail in Section 1.8.

1.3 GLYCOLYSIS IN THE ERYTHROCYTE

Glycolysis in the mature erythrocyte, unlike all other cells in the body, is anaerobic, irrespective of whether oxygen is present or not (Surgenor, 1975). The glycolytic pathway, which is also called the Embden-Meyerhof pathway (EMP), and the connected pentose shunt pathway, are shown in Fig. 1.1. Glucose metabolism is critically important to the erythrocyte, first to provide the energy for various functions, and second, to modulate haemoglobin function. Murphy (1960) has studied the proportion of substrate which flows through the pentose shunt under laboratory conditions designed to simulate *in vivo* conditions as closely as possible, and concludes that 10% normally goes through the pentose shunt, the other 90% passing down the EMP. The

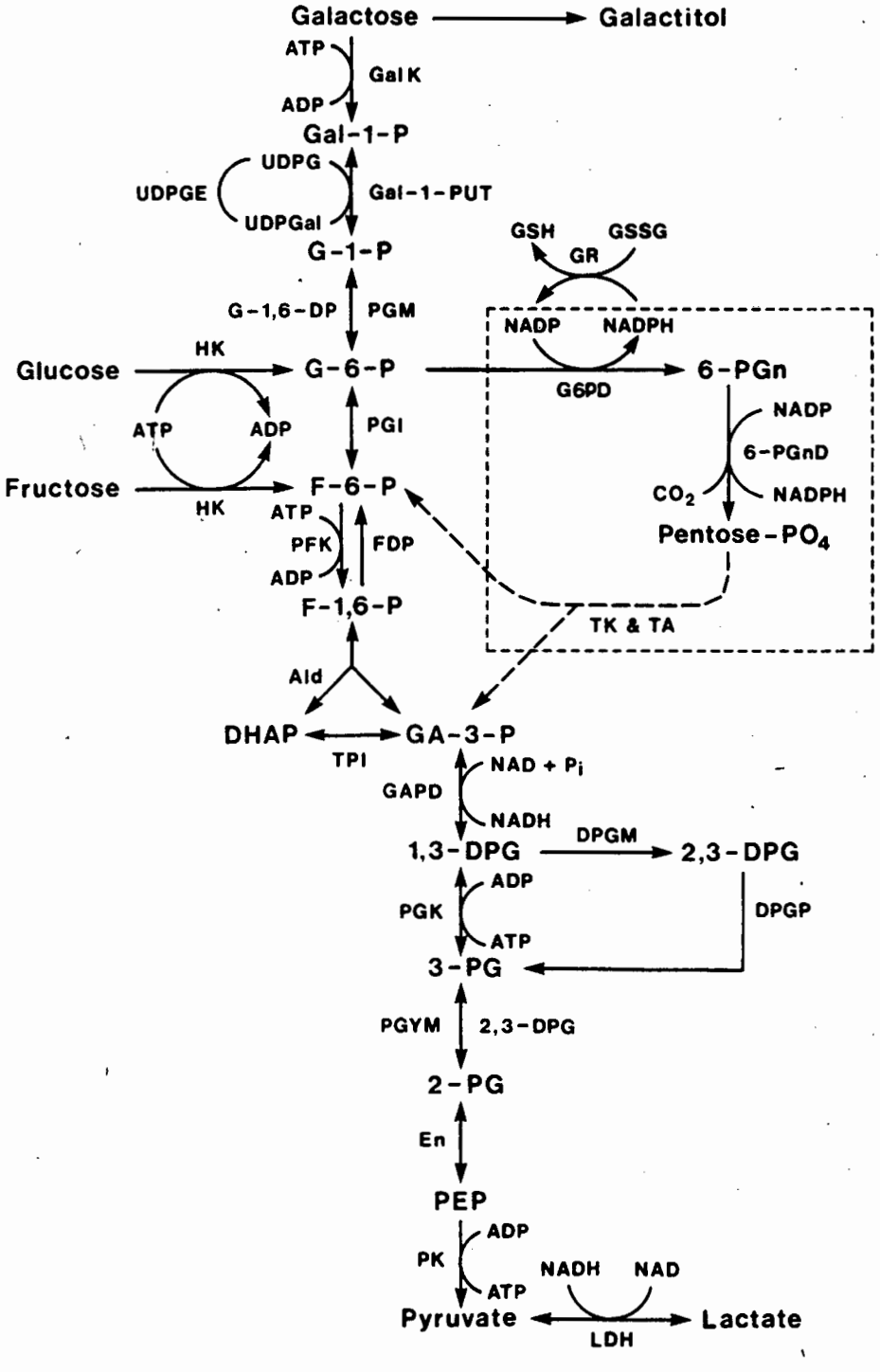
Figure 1.1. The major pathways of carbohydrate metabolism in mature mammalian erythrocytes (Brewer, G.J., 1974).

Key

The pentose shunt is enclosed in the dotted box. The arrows represent enzymatic steps.

Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate; Ald, fructoaldolase; DHAP, dihydroxyacetone phosphate; 1,3-DPG, 1,3-bisphosphoglycerate; 2,3-DPG, 2,3-bisphosphoglycerate; DPGM, bisphosphoglycerate mutase; 2,3-DPGP, bisphosphoglycerate phosphatase; En, enolase; FDP, fructose-1,6-bisphosphate; F-6-P, fructose-6-phosphate; G-1,6-DP, glucose-1,6-diphosphate; G-1-P, glucose-1-phosphate; G-6-P, glucose-6-phosphate; G-6-PD, glucose-6-phosphate dehydrogenase; GA-3-P, glyceraldehyde-3-phosphate; GAPD, glyceraldehyde-3-phosphate dehydrogenase; GalK, galactokinase; Gal-1-P, galactose-1-phosphate; Gal-1-PUT, galactose-1-phosphate uridyl transferase; GSH, reduced glutathione; GSSG, oxidised glutathione; HK, hexokinase; LDH, lactate dehydrogenase; NAD and NADH, oxidised and reduced nicotinamide adenine dinucleotide, respectively; NADP and NADPH, oxidised and reduced nicotinamide adenine dinucleotide, respectively; NADP and NADPH, oxidised and reduced nicotinamide adenine dinucleotide phosphate, respectively; PEP, phosphoenol pyruvate; PFK, phosphofructokinase; 2-PG, 2-phosphoglycerate; 3-PG, 3-phosphoglycerate; 6-PG, 6-phosphoglycerate; 6-PGD, 6-phosphoglycerate dehydrogenase; PGI, phosphohexose isomerase; PGK, 3-phosphoglycerate kinase; PGM,

6-phosphogluconate dehydrogenase; PK, pyruvate kinase; TA, transaldolase; TK, transketolase; TPI, triosephosphate isomerase; UDPG, uridine 5'-diphosphate glucose; UDPGal, uridine 5'-diphosphate galactose; UDPGE, uridine 5'-diphosphate glucose epimerase.



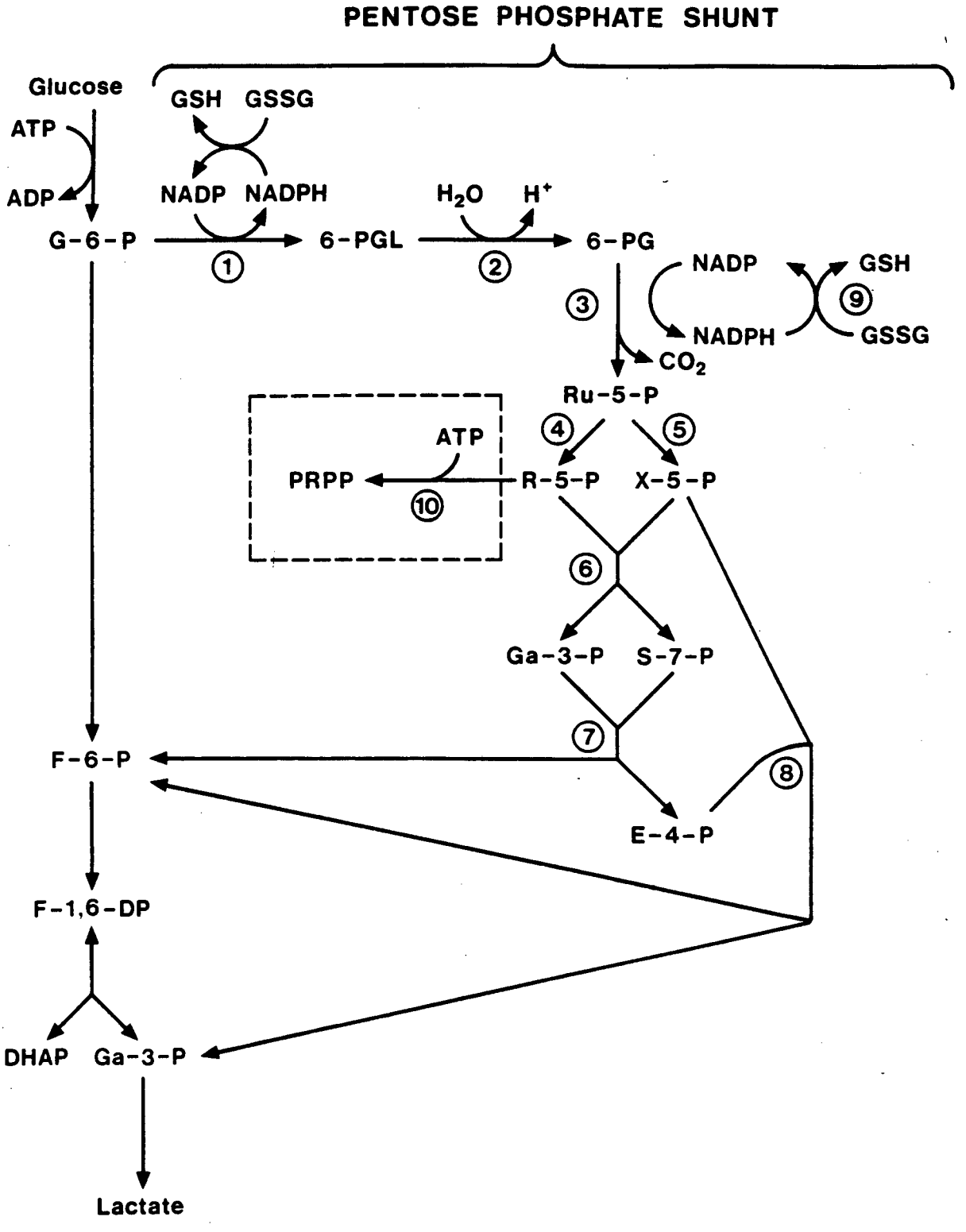
proportion of carbohydrate substrate passing through the pentose shunt can increase dramatically as a result of different kinds of stimuli, the 10% figure representing, then, a form of basal traffic. The known general functions of the pentose shunt in the erythrocyte are reduction of nicotinamide adenine dinucleotide phosphate (NADP) and hence indirectly glutathione (GSSG), and the generation of 5'-phosphoribosyl alpha-1-pyrophosphate (PRPP).

1.4 THE PENTOSE PHOSPHATE SHUNT AND PRPP PRODUCTION

The major features of the pentose shunt are shown in Fig. 1.2 (Eaton and Brewer, 1974). Production of PRPP is a function of the pentose shunt in the mature erythrocyte. It may be used by this cell for the synthesis of adenine nucleotides. Herschko *et al*. (1969) have demonstrated that the synthesis of PRPP by phosphoribosyl pyrophosphate synthetase (PRPP synthetase) is controlled by a balance between naturally present inhibitors and activators. These authors reported that inorganic phosphate (Pi) was an effective activator of PRPP synthesis and that ADP, GDP and 2,3-DPG were potent inhibitors. The activity of PRPP synthetase within the whole cell, then, will be determined by the nett effect of these activating and inhibiting compounds.

Figure 1.2. The pentose shunt (Eaton and Brewer, 1974). Reactions are drawn unidirectionally although many are reversible. Circled numbers refer to the following enzymes: (1) glucose-6-phosphate dehydrogenase; (2) 6-phosphogluconolactonase; (3) 6-phosphogluconate dehydrogenase; (4) ribose-5-phosphate isomerase; (5) xylulose-5-phosphate epimerase; (6) transketolase; (7) transaldolase; (8) transketolase; (9) glutathione reductase; (10) 5′-phosphoribosyl-1-pyrophosphate synthetase. Abbreviations (see Fig. 1.1 for abbreviations not appearing here): E-4-P, erythrose-4-phosphate; 6-PG, 6 phosphogluconate; 6-PGL, 6-phosphogluconolactone; PRPP, 5′-phosphoribosyl-1-pyrophosphate; R-5-P, ribose-5-phosphate; Ru-5-P, ribulose-5-phosphate; S-7-P, sedoheptulose-7-phosphate; X-5-P, xylulose-5-phosphate.

Fig. 1.2


PENTOSE PHOSPHATE SHUNT
Glucose
ATP
ADP
G-6-P
GSH GSSG
NADP NADPH
①
6-PGL
H_2O H^+
②
6-PG
③
CO_2
NADP
NADPH
GSH
GSSG
⑨
Ru-5-P
④
⑤
ATP
PRPP
⑩
R-5-P
X-5-P
⑥
Ga-3-P
S-7-P
⑦
⑧
F-6-P
E-4-P
F-1,6-DP
DHAP
Ga-3-P
Lactate

1.5 PHOSPHORIBOSYLPYROPHOSPHATE (PRPP) SYNTHETASE AND PHOSPHORIBOSYLPYROPHOSPHATE

PRPP synthetase from all sources has an absolute requirement for Pi for its activity (Wong and Murray, 1969). The apparent Km (or Ka) of Ehrlich ascites cell enzyme for Pi is 3.3 mM (Roth et al., 1974a,b). Removal of Pi leads to immediate and complete loss of enzyme activity (Fox and Kelley, 1971a and b). In Sperling's view, the rate of PRPP synthesis in liver is largely regulated by Pi (Sperling, 1977). The enzyme also requires magnesium or manganese. With purified human PRPP synthetase the Km value for ribose-5-phosphate (R-5-P) is 3.3×10^{-5} M, and for magnesium ATP is 1.4×10^{-5} M (Fox and Kelley, 1972).

The smallest native form of PRPP synthetase from human erythrocytes has a molecular weight of 60,000 and consists of two subunits of equal molecular weight (Fox and Kelley, 1971a). In the presence of saturating concentrations of ATP and magnesium chloride, the enzyme associates into two heavy forms with molecular weights of about 720,000 and 1.2 million. Ribose-5-phosphate does not alter the aggregation state of the enzyme. The associated enzyme appears to be the active form. In addition to the heterogeneity of molecular size, human PRPP synthetase exhibits striking electrophoretic heterogeneity. Electrophoretic variants of the erythrocyte enzyme were found in 2.5 percent of 200 subjects. In addition, human organs obtained at autopsy disclosed a unique electrophoretic mobility for nearly each

in human cells (Fox and Kelley, 1971b). However, the Ki of the enzyme for 2,3-DPG is approximately equal (5.3 mM) to its concentration in erythrocytes. Therefore, 2,3-DPG may participate in the control of PRPP synthesis.

A large number of nucleotides, including AMP, ADP, GDP, GTP, IDP, ITP, TDP, NAD, NADPH and FAD inhibit PRPP synthetase by a third mechanism which is non-competitive with respect to both magnesium-ATP and R-5-P (Fox and Kelley, 1972; and Green and Martin, 1973). In general terms the di- and triphosphate derivatives are more potent inhibitors than the monophosphates. This group of inhibitors has a low affinity for the enzyme, presumably binds at a single site, and regulates by a mechanism called "heterogeneous metabolic pool inhibition". This term means that the degree of inhibition depends on total nucleotide concentration and is largely independent of specific nucleotide composition. Atkinson (1968) postulates that the activity of a biosynthetic reaction is controlled by the "energy charge" of the cell (Atkinson, 1968), as well as by feedback inhibition:

$$\text{"Energy charge"}: = \frac{\text{ATP} + \tfrac{1}{2}\text{ADP}}{\text{ATP} + \text{ADP} + \text{AMP}}$$

This concept predicts that the synthesis of PRPP will be inhibited by nucleoside diphosphates and monophosphates, irrespective of specific feedback effects.

Steady state levels of PRPP will be determined by the balance achieved between rates of production and of utilization. Data on intracellular concentrations of PRPP are fragmentary. Values in normal human erythrocytes range from 2 to 7 micromolar (Green and Seegmiller, 1969; Fox et al., 1970, and Van Maris et al., 1980).

Methylene blue (reduced) will raise the intracellular concentration of PRPP in human fibroblasts in tissue culture, and in human erythrocytes in vitro (Wyngaarden and Kelley, 1983), presumably by accelerating the regeneration of NADP in the oxidative pathway of glucose metabolism and thereby stimulating the rate of production of R-5-P. In addition, ethanol has been shown to raise PRPP concentrations in isolated mouse hepatic cells (Nishida et al., 1979).

PRPP concentration values are elevated in cells with deficient hypoxanthine-guanine phosphoribosyltransferase (HPRT) activity (Rosenbloom et al., 1968). HPRT-deficient fibroblasts show accelerated rates of purine biosynthesis. Intracellular PRPP concentrations may be reduced by stimulating PRPP consumption with allopurinol, orotic acid, adenine or 2,6-diaminopurine (Wyngaarden and Kelley, 1983). Such measures reduce the rate of purine biosynthesis de novo, except in HPRT-deficient cells which have a surfeit of PRPP.

1.6.1 Membrane transport of anions in erythrocytes: the band 3 protein

Anion transport across the erythrocyte membrane is not energy-dependent and is facilitated by a major integral membrane protein known as band 3 because of its electrophoretic properties in SDS polyacrylamide gel (Fairbanks et al., 1971). The structural and kinetic properties of this general anion transporter have been extensively investigated (Cabantchik and Rothstein, 1974; Jenkins and Tanner, 1975; Ho and Guidotti, 1975; Steck et al., 1976; Markowitz and Marchesi, 1981; and Macara and Cantley, 1981) and few topics have been so thoroughly reviewed in recent years as anion exchange in human erythrocytes (see for example, Rothstein et al., 1981; Brahm, 1986; Knauf, 1979; Passow, 1986). Some evidence that the band 3 protein is the anion carrier is provided by the fact that erythrocyte membranes depleted of most proteins except band 3, retain anion transport and the transport in these membranes has the same properties as that present in intact erythrocytes (Ross and McConell, 1977). Furthermore, band 3 has been incorporated into phospholipid vesicles that were then able to transport anions (Ross and McConnell, 1977).

Rapid transport of anions is a specialized function of the erythrocyte membrane that enables these cells to play an important role in blood carbon dioxide transport. Instead of band 3, the name capnophorin was proposed by Wieth and

Bjerrum (1983) to characterise the physiological function of the transport system: "Kapnos is the Greek word for smoke. Carbon dioxide is the fume of oxidative metabolism, and hypercapnia is the well-known pathophysiological consequence of impaired CO_2 elimination from the organism". CO_2 formed in the tissues rapidly diffuses into erythrocytes where it is converted to H^+ and HCO_3^- by carbonic anhydrase. The protons are buffered by haemoglobin, and the band 3 protein in the erythrocyte membrane mediates the (non-energy dependent) exchange of cellular HCO_3^- for plasma Cl^- (Hamburger, 1891). This removes the product of the carbonic anhydrase reaction and permits CO_2 to be stored in the form of plasma-bicarbonate, thus greatly increasing the CO_2-carrying capacity of the blood (Wieth *et al.*, 1982), and explaining the relatively high Cl^- concentration of 50 mM found in erythrocytes (Caraway, 1962).

For this process to be physiologically significant, the exchange must take place within the time of passage of an erythrocyte through a pulmonary capillary, which varies from 0.7 seconds at rest to 0.3 seconds during exercise (Wieth, 1979; Wieth *et al.*, 1982). Chloride and bicarbonate self-exchange is very fast with half-times of about 40 to 50 milliseconds at 37°C (Tosteson, 1959; Brahm, 1977), more than a million times faster than the corresponding rates of cation exchange (Wieth, 1972). Even so, the rate of exchange with physiological gradients of Cl^- and HCO_3^- is, nevertheless, only barely sufficient to permit anion

equilibration under conditions of exercise, and the anion exchange actually becomes rate-limiting for CO_2 transport under exercise (Wieth et al., 1982). Perhaps it is because of the need to maximise the transport rate that the erythrocyte contains such a large amount of band 3 protein: there are estimated to be 800 000 to 1 200 000 molecules of the band 3 protein per cell (Knauf, 1979), and it is the most abundant protein of the erythrocyte membrane, constituting some 25 to 30 per cent of the total membrane protein.

The low permeability to cations and a specialised system for rapid exchange of anions (Wieth, 1972; George Fortes, 1977) in conjunction with an active cation pump system, provides a means of regulating cell volume at low energy cost (Tosteson, 1964).

On SDS polyacrylamide gel electrophoretograms the 95 kDa band 3 protein extends over a broad molecular weight range of at least 10 kDa. This diffuse appearance of the band disappears after suitable treatment with glycosidases (Mueller et al., 1979; Fukuda et al., 1979; Jennings, 1984), in agreement with the suggestion that it is largely due to the heterogeneity of the carbohydrate moiety of the molecule (Drickamer, 1978).

Besides the mediation of anion exchange, other possible functions of the protein have been considered. There are suggestions that it is also involved in the transport of

water (Benz et al., 1984; Chasan et al., 1984; Yoon et al., 1984), sugars (Shelton and Langdon, 1983; Acevedo et al., 1981), and cations (Solomon et al., 1983), but so far no general agreement seems to exist about their validity. A consensus has however developed with regard to sugar transport: it is accomplished by the band 4.5 protein (Deziel et al., 1984). Other functions could possibly be related to the capacity of the glycoprotein's outward-facing carbohydrate moiety to react with lectins (Findlay, 1974; Tanner and Anstee, 1976) and its inward-facing N-terminal segment to combine with haemoglobin (Salhany et al., 1980) and intracellular enzymes (Strapazon and Steck, 1977; Yeltman and Harris, 1980). There seems to be little doubt that it plays an important part in anchoring the constituents of the cytoskeleton in the lipid bilayer (Bennett and Stenbuck, 1979, 1980; Hargreaves et al., 1980). These observations and the uncertainties about possible additional functions explain why the prosaic term "band 3" is usually preferred to "anion transport protein" or "capnophorin".

1.6.2 Structure of band 3

By proteolytic digestion in situ (Drickamer, 1977) it is possible to split the band 3 protein into a number of well-defined fragments; internal trypsin for example splits off a hydrophilic 42 kDa piece (Lepke and Passow, 1976) which is released from the membrane (Steck, 1978) while the remaining 55 kDa piece stays in the bilayer. External trysin produces no cleavage. When erythrocytes are treated with external

chymotrypsin two fragments of 35 kDa and 60 kDa are formed that both remain associated with the membrane.

The hydrophobic 55 kDa fragment and the hydrophilic 42 kDa fragment that are obtained after cleavage by internal trypsin, form two rather independent domains of the band 3 protein with different functions (Snow et al., 1981). The former is essentially responsible for anion transport and the latter for the binding of cytoskeletal proteins and, possibly, other intracellular proteins.

Peptide maps of the 42 kDa water-soluble fragment of band 3 have been published (Fukuda et al., 1978) and the amino acid sequence of the 201 N-terminal amino acid residues has been reported (Kaul et al., 1983).

It is noteworthy that in spite of the immense efforts made in many competent laboratories, only minor pieces of the amino acid sequence of the hydrophobic transport-related domain of the band 3 protein have been established. One of the major reasons for the slow progress was due to the hydrophobicity of many of the cleavage products, which made it difficult to separate them from one another. Kopito and Lodish (1985) have however succeeded in isolating cDNA clones of the full length of the message for mouse band 3 protein and thus have managed to obtain the complete amino acid sequence for murine band 3. Analysis of the sequence of this murine band 3 reveals that the protein possesses 12 membrane-spanning

regions, 7 of which form amphipathic helices which could cluster around an aqueous "pore". This structure contains a gating mechanism, possibly formed by guanidino and carboxyl groups, which prevents free diffusion and which catalyses a strict one-for-one exchange of anions (Wieth and Brahm, 1985; Knauf, 1979).

A discussion on the biosyntheis, post-translational modification and membrane insertion of band 3 is beyond the scope of this thesis. The interested reader is however referred to publications from two independent research groups (Braell and Lodish, 1981 and 1982; Lodish and Braell, 1982; Sabban et al., 1980, 1981; see also a review by Sabatini et al., 1982)

The C-terminal 55 kDa domain of band 3 is anchored in the aqueous phase of the outer medium by the carbohydrates that are attached to it. The N-terminal end resides in the hydrophilic 42 kDa domain which protrudes into the cytoplasm at the inner membrane surface; hence, anion transport cannot be accomplished by rotational diffusion of the transport protein across the lipid bilayer (Passow, 1986).

Chemical probes which react specifically with the band 3 protein have afforded much information on the structure and function of the anion transport system (for review, see Catantchik et al., 1978). Among these probes, stilbene disulfonate derivatives have been shown to bind covalently to

the membrane-spanning portion of band 3 and competitively inhibit self exchange of anions (Grinstein et al., 1978). Certain other probes such as NAP-taurine and alpha-cyano-4-hydroxycinnamate and their analogues, when added outside the membrane, interact with an allosterically linked "modifier site" (Dalmark, 1976) to cause non-competitive inhibition of anion transport (Knauf et al., 1984; Halestrap and Denton, 1975). These chemical modification studies are regarded as indicating that the membrane spanning portion of band 3 forms the anion channel and contains a substrate-binding site and a modifier site within it.

1.6.3 Mechanism of anion transport by band 3.

The single transport site on band 3 is alternating in nature and proceeds via a ping-pong mechanism (Gunn and Fröhlich, 1979; Falke and Chan, 1985). It oscillates between two different conformations, alternately exposed to opposite sides of the membrane, one in which internal (cytoplasmic) anions can bind to the transport site, and another in which external anions can bind. The transport site can only cross the membrane when it is occupied by substrate anion; thus when an anion gradient is imposed across the membrane the transport site accumulates on the side of the membrane exposed to low anion concentration (Jennings, 1982). Said in another way, a characteristic of the electroneutral exchange mediated by band 3 is that flow of an anion from one side of the membrane (cis) to the other (trans) is strongly

dependent on the presence of transportable anions at the trans side (Hoffmann, 1986).

Using stilbene disulphonates (Knauf et al., 1977) and studies on the temperature (Fröhlich et al., 1983) and pH dependence (Knauf et al., 1983a,b) of the various flux components, Knauf (1986) concluded that anion transport across the erythrocyte membrane can be dissected into three components, two of which are mediated by band 3 protein and another that is probably not. Band 3 seems to mediate (1) the physiologically important electrically silent anion exchange process described above and (2) a relatively insignificant diffusive flow that contributes to the small conductance seen. The remaining conductance appears to come from a band 3-independent conductance pathway.

1.6.4 Anion specificity

The kinetics of mono- and divalent anion transport are similar in many respects. Equilibrium exchange shows saturation kinetics with self-inhibition at high substrate concentration (Barzilay and Cabantchik, 1979). Both mono- and divalent anion transport have the same unusually high activation enthalpies, and in addition they compete with each other for exchange (Schnell et al., 1977). Finally, a large number of inhibitors produce the same fractional inhibition of mono- and divalent transport (Ku et al., 1979).

Nevertheless, there exist two important differences: (1) The rate of transport of divalent anions is many orders of magnitude lower than that for monovalent anions and (2) differences in pH dependence. Details of these differences follow.

Studies of self-exchange of mono- and divalent anions (Passow, 1969; George Fortes, 1977; Brahm, 1986) have not only confirmed the selectivity of the erythrocyte membrane for anions versus cations but have also demonstrated marked differences in the transport rates for various anions. The anions can be ranked according to their relative self-exchange rates as:

$$HCO_3^- > Cl^- > Br^- > F^- > SCN^- > I^- >> SO_4^{2-} \text{ and } PO_4^{2-}.$$

At physiological pH chloride equilibrates with a half-time of 210 msec at room temperature (Tosteson, 1959; Dalmark, 1975) and SO_4^{2-} and PO_4^{2-} equlibrate with half-times between minutes and hours at 37°C (Passow, 1969). The penetration rates are not simply related to the relative apparent affinities of the anions for the transport system, as indicated by the fact that I^- is transported about 300 times more slowly than HCO_3^-, although the apparent affinity of the two anions for the transporter is similar (Dalmark ,1976; Wieth, 1979). It should be noted that OH^- is not included in the sequence as recent experiments have shown that this anion is not transported by the anion transport system to any measurable degree (Wieth et al., 1980). The apparent flux of OH^- across the membrane is in fact due to the transport of

bicarbonate ions which, by entering the Jacobs-Stewart cycle (Jacobs and Stewart, 1942) alter the hydroxyl ion concentration so that the distribution of hydroxyl ions across the membrane equals the distribution of the monovalent anions.

Measurements of pH dependence of mono- and divalent anion fluxes have revealed an interesting difference. Whereas the flux of divalent anions such as sulphate (Schell et al., 1977, 1981) and phosphate (Deuticke, 1973) has a maximum between 6.0 and 6.5 and decreases in the range between 6.5 and 7.5, the flux of monovalent anions such as chloride and iodide increases between pH 6.5 and 7.5 and has a broad maximum around pH 7.8 (Gunn 1973; Gunn et al., 1973).

To explain the pH dependent behaviour Gunn (1972, 1973) has proposed a model in which the carrier (band 3) can be titrated by hydrogen ions to form monovalent and divalent carriers. At high pH values, the carrier is in the uncharged form and does not carry ions. Titration of the medium from pH 9.5 to 7.8 causes an increasing number of monovalent anion carriers to be formed, and the flux of monovalent anions across the membrane increases accordingly. If the pH is lowered past 7.4, the monovalent carriers are converted into divalent carriers; monovalent anion flux decreases and divalent flux increases. Below pH 6.5 the carrier is titrated to the triply charged form which does not carry anions. Triply charged anions (e.g. citrate) are

therefore unable to cross membranes (Passow, 1986). The reactions of anions at the interface are considered to be at equilibrium, and the rate limiting step is the translocation of the neutral carrier-anion complex from one surface to the other. It is further assumed that if the monovalent ions combine with the divalent carrier, as they appear to do at low pH, the complexes formed are translocated very slowly (Sachs et al., 1975).

There are certain objections to Gunn's model of a titratable carrier, the details of which are beyond the scope of this overview. The interested reader is however referred to Passow's (1986) excellent review article. Suffice it to say that to date, Gunn's model is the one which can best explain experimental observations, notwithstanding the difficulties in interpreting the data of phosphate fluxes: over the physiological range there are variations of the ratio between monovalent and divalent ions. Runyon and Gunn (1984) and Berghout et al. (1985) have reported conflicting observations on the flux of monovalent and divalent phosphate ions.

1.6.5 Transport of organic anions

Small mono- and divalent organic anions are transported across the erythrocyte membrane by the general anion transporter (the band 3 protein) if of the correct configuration (Deuticke, 1973; Knauf, 1979; Nanri et al., 1983 and see Motais, 1977 for review). Molecules with a carbon chain length greater than four are not transported

unless amphiphilic in structure. The rates of transport of these organic anions are some hundreds to millions of times slower than for chloride and bicarbonate ions; the discrepancy being ascribed to differences in size, polarity and configuration.

1.6.6 Anion transport in non-erythroid cells

There are three general mechanisms by which anions cross plasma membranes of vertebrate cells (Hoffmann, 1986):
(i) electrodiffusion, which must respond to the electrochemical potential of the anion at the two sides of the membrane, (2) electroneutral exchange mediated by the band 3 protein, and (3) the coupled flow (cotransport) systems of anions and cations which can be classified as "secondary active" transport systems since they depend on electrochemical gradients generated by the enzymatic hydrolysis of ATP.

The large amounts of band 3 protein contained in the human erythrocyte membrane bestows upon it the capacity to transport anions rapidly across its membrane to fulfill, in part, its physiological role. Kay *et al*. (1983) and Drenckhahn and Zinke (1984) have demonstrated that polypeptides sharing common antigenic determinants with erythrocyte band 3 are present in non-erythroid cells, as determined by immunofluorescence, immunoelectronmicroscopy and immunoautoradiography. It would however appear that non-erythroid cells contain much less band 3 than is present

in erythrocytes. Furthermore, these polypeptides, which are immunologically related to band 3 are mostly in the form of a 42 kDa breakdown product of band 3 (Kay et al., 1983) which is unable to transport anions in the rapid one-for-one exchange fashion mediated by the intact 95 kDa molecule. Fibroblast plasma membranes, for example, contain only traces of the intact 95 kDa band 3 protein (Drenckhahn et al., 1984). Hepatocytes may be a notable exception as Cheng and Levy (1980) have demonstrated a 97 kDa anion transport protein in rat hepatocytes.

1.7 TRANSPORT OF PURINES ACROSS THE ERYTHROCYTE MEMBRANE

Erythrocyte uptake[1] of nucleosides and nucleobases can be viewed as a two-component system, comprised of symmetrical, carrier-mediated, non-concentrative transport[2] across the cell membrane coupled with simple, irreversible Michaelian phosphorylation within the cell (Plagemann and Wohlhueter, 1980; Wohlhueter and Plagemann, 1980). Most experimental results to date are consistent with the independent operation of the two steps (Plagemann, 1986).

Human erythrocytes possess only a single nucleoside/ nucleobase transport system with broad substrate specificity (Overgaaard-Hansen and Lassen, 1959; Lassen and Overgaard-

[1] "Uptake" denotes the total intracellular accumulation of radioactivity from exogenous substrate regardless of metabolic conversions.

[2] "Transport" denotes the transfer of unmodified substrate across the cell membrane as mediated by a saturable selective carrier.

Hansen, 1962a; Lassen and Overgaard-Hansen, 1962b; Oliver and Paterson, 1971; Cass and Paterson, 1972, 1973). Working with monolayer cultures of non-proliferating rabbit polymorphonuclear leukocytes, Taube and Berlin (1972) found that these cells also possessed a single nucleoside transport system. Plagemann and Richey (1974) however, found several nucleoside transport systems in cultured animal cells and whether or not several nucleoside transport systems operate in non-erythroid cells has not yet been finally settled (Plagemann et al., 1985a and b).

The transport of nucleosides/nucleobases across the erythrocyte membrane is facilitated by both saturable and non-saturable carrier systems (Lassen, 1967) and is rapid, equilibrium being reached within a minute, even at millimolar concentrations (Lassen, 1967; Plagemann and Wohlhueter, 1980). Until recently, the transport process was thought to be by facilitated diffusion and not energy dependent (Plagemann and Wohlhueter, 1980). However, it has recently come to light that there is a second nucleoside transport system which is concentrative and sodium-dependent (Jakobs and Paterson, 1986; Vijayalakshmi and Belt, 1988).

1.8 ERYTHROCYTE PURINE METABOLISM

Figure 1.3 serves as a focus for this section.

The purine nucleotides of mature mammalian erythrocytes undergo turnover as shown by incubation studies carried out by a number of authors (Lowy et al., 1960; Bishop et al., 1959), as well as by in vivo studies (Lowy et al., 1958; Bishop, 1961; Mager et al., 1966). So-called de novo synthesis of purines does not appear to take place in mature

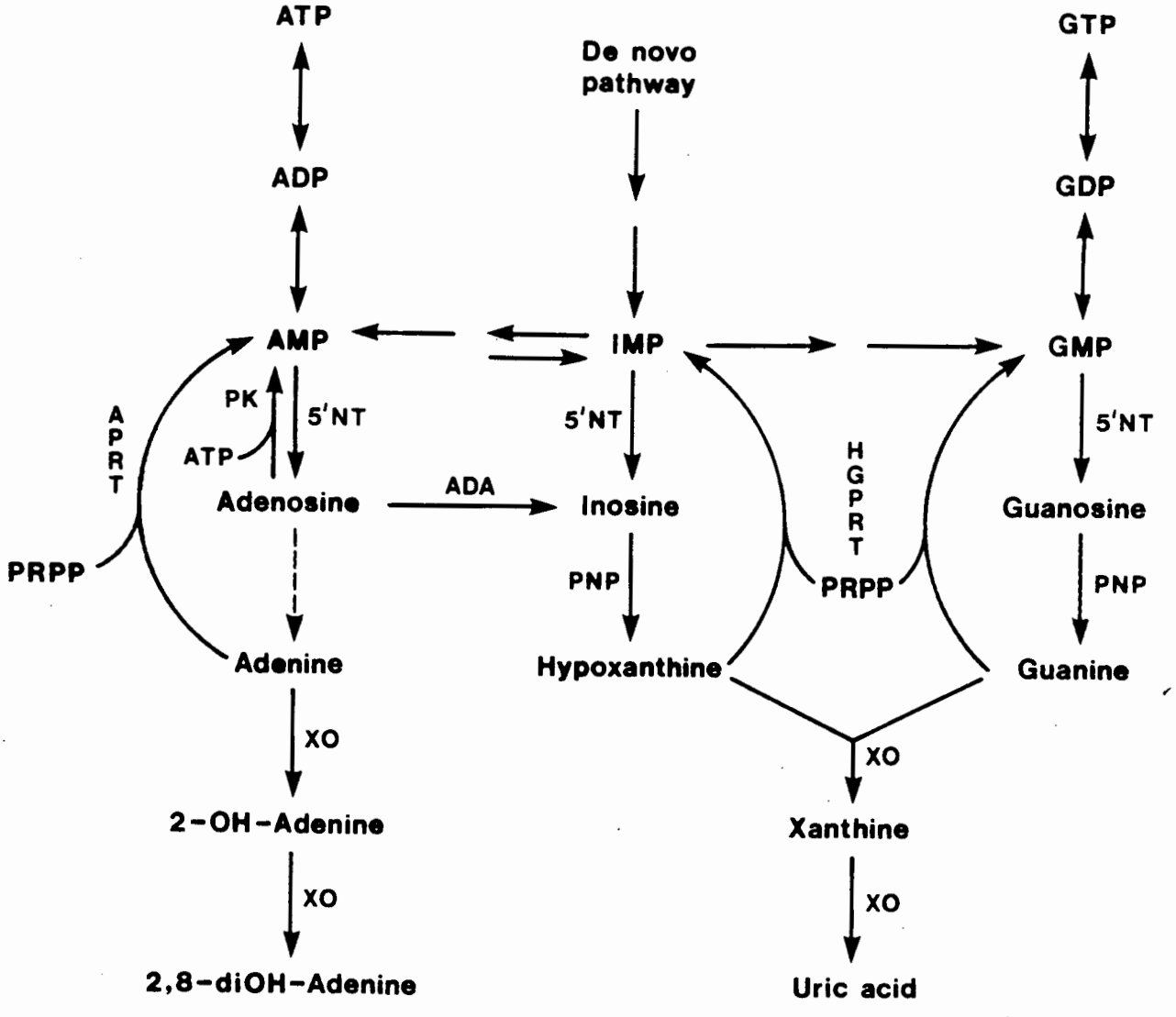


Figure 1.3. Outline of purine metabolism in the erythrocyte. ADA, adenosine deaminase; APRT, adenosine phosphoribosyl transferase; HGPRT, hypoxanthine-guanine phosphoribosyl transferase; 5'NT, purine 5'-nucleotidase; PK, purine kinase; PNP, purine nucleoside phosphorylase; XO, xanthine oxidase

human erythrocytes because one of the enzymes of the last portion of the de novo pathway (adenylosuccinate synthetase) is not present in these cells (Lowy et al., 1962). (Adenylosuccinase, another enzyme in the terminal part of the de novo pathway, was also believed to be absent in mature human erythrocytes (Lowy et al., 1962), but Jaeken and Van den Berghe (1984) have subsequently demonstrated its presence.) However, the rabbit erythrocyte does have the enzymes of the last portion of the de novo pathway (Lowy et al., 1960, 1961a, 1961b). This allows the rabbit erythrocyte to use the adenine (and guanine) precursor, 5-amino-1-ribosyl-4-imidazole carboxamide. Preiss and Handler (1958), Zeitlin-Beck et al. (1964), Soshani et al. (1965) and Mager et al. (1966) have shown that the nucleotide derivatives of adenine, guanine and hypoxanthine each exhibit a different and characteristic rate of renewal via a highly efficient salvage mechanism which uses the preformed purines as nucleotide precursors.

It is noteworthy that the biological half-life of adenine is about ten-fold longer in human than in rabbit erythrocytes (Zeitlin-Beck et al., 1964), notwithstanding the essential similarity in the overall patterns of purine turnover displayed by these cells. It appears likely that this disparity between the erythrocytes of the two species is inherent in the different extent of contribution of exogenous purines to their adenine pools, as determined by the capacity for converting hypoxanthine to adenine which is negligible in the human and substantial in rabbit erythrocytes (Lowy et al., 1961a; Hershko et al., 1963; Lowy et al., 1962).

The unique status of hypoxanthine in the metabolism of erythrocyte purines is reflected in its exclusive intracellular appearance in the form of IMP, thus differing from both adenine and guanine which are represented in the cell predominantly by their respective nucleoside triphosphates (Mager et al., 1966). Hypoxanthine is further distinguished by its particularly rapid rate of clearance from the erythrocyte, as well as by the low level of its normal intracellular pool (Bishop et al., 1959). This distinctive behaviour of hypoxanthine is consistent with its role both as an end product of the metabolism of purine nucleotides in the erythrocyte (Bishop et al., 1959; Hershko et al., 1963), and as a mediator in the postulated two way traffic of purines between the liver and peripheral tissues, in which the erythrocyte may serve as the transporting vehicle (Henderson and Le Page, 1959; Zeitlin-Beck, 1964; Soshani et al., 1965; Murray, 1971; Pritchard et al., 1975; Fox and Kelley, 1978). The turnover of adenine nucleotides in erythrocytes can proceed via two pathways, both of which yield hypoxanthine: AMP IMP Inosine Hypoxanthine and AMP Adenosine Inosine Hypoxanthine (Plagemann et al., 1985b), but the extent to which these function in erythrocytes in the body and might be influenced by O_2 tension (see discussion in Chapter 2) and aging of the cells is unclear. Any adenosine that is generated in the second pathway might be rapidly rephosphorylated, except perhaps in aging cells, but the hypoxanthine that is formed will be released, because of the limited capacity of the cells to

phosphoribosylate it at physiological phosphate concentrations. Hypoxanthine may be the main purine source for other body cells which exhibit a greater salvage capacity than erythrocytes under these conditions.

In view of the absence of de novo synthesis, the mechanism by which the human erythrocyte turns over its adenine nucleotides is not completely clear. By examining Fig. 1.3 one can see that AMP can be synthesized from adenine through the action of APRT and PRPP, or from adenosine via adenosine kinase and ATP. The synthesis of PRPP in the erythrocyte has been discussed in section 1.5.

Adenine is not detectable in the plasma of normal humans (Brewer, 1974). Henderson and Le Page (1959) have suggested that as the erythrocyte passes through the liver it picks up adenine, perhaps with such avidity that detectable levels are not seen in peripheral blood. The addition of adenine to blood before storage has been shown to be beneficial in terms of maintaining ATP levels and improving the survival of long-stored erythrocytes after transfusion (Brewer, 1974). It is likely that this benefit accrues from the adenine-facilitating synthesis of new adenine nucleotide. Plagemann (1986), however, has shown that adenine is probably of little use to erythrocytes because it is poorly salvaged under physiological conditions.

Meyskens and Williams (1971) make a case for an important role for adenosine and adenosine kinase in the synthesis of AMP in human erythrocytes They point out that uric acid production is normal in patients with APRT deficiency, which suggests that this route is not too important for adenine nucleotide synthesis, at least at the whole body level. Meyskens and Williams (1971) provide kinetic data for adenosine kinase and outline mechanisms for its possible regulation of adenine nucleotide synthesis. This view is supported by Plagemann (1986) who found that adenosine is the main purine source for salvage in the human erythrocyte. Again, a source of adenosine is required and Lowy and Lerner (1974) and Pritchard et al. (1975) have demonstrated a role for liver adenosine in the renewal of the adenine nucleotides in human and rabbit erythrocytes.

Since ATP is involved in various aspects of glycolytic regulation in the red cell, the mechanisms by which adenine or adenosine is provided to the circulating erythrocyte are of considerable interest. That is, it seems possible that the availability of these substances, if they are normally supplied exogenously to the cell, could have an important role in determining the level of erythrocyte ATP as has been suggested by Syllm-Rapaport et al., (1969) for adenine. Increases in ATP might result from increased availability of adenine or adenosine and would be expected to activate hexokinase and possibly to inhibit phosphofructokinase. In

this manner, the exogenously supplied purines could play an important role in regulating overall glycolysis.

The nucleoside inosine has an effect upon erythrocyte metabolism but does not appear to contribute to AMP synthesis in the mature erythrocyte (Brewer, 1974). Inosine can penetrate the erythrocyte membrane and is then converted to ribose-1-phosphate and hypoxanthine by purine nucleoside phosphorylase. The ribose-1-phosphate is converted to ribose-5-phosphate which then enters the pentose phosphate shunt and provides substrate which re-enters the EMP in the usual manner. In this way phosphorylated substrate is obtained, bypassing the hexokinase and phosphofructokinase reactions (Fig. 1.1). With the use of inosine it is possible to increase substrate flow into the glycolytic pathway both in vitro and in vivo. However, the buildup of 2,3-DPG and ATP by inosine alone is not nearly as great as with the use of inosine and Pi together (Sugerman et al., 1972). In the absence of Pi there is an accumulation of fructose-1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde-3-phosphate which probably indicates a lack of Pi at the glyceraldehyde-3-phosphate dehydrogenase step. With the addition of Pi, substrate flows down to the lower part of the pathway and 2,3-DPG levels, and to a certain extent ATP levels, are increased. These effects can be increased even further by the addition of pyruvate which apparently generates additional NAD by reversing the lactate dehydrogenase reaction (Sugerman et al., 1972). The NAD is

required at the glyceraldehyde-3-phosphate dehydrogenase step. Inosine has been found to be particularly effective in rejuvenating blood after prolonged storage in which ATP has reached quite low levels (Brewer, 1974). Owing to the low levels of ATP, phosphorylation of glucose by hexokinase is ineffective and inosine provides substrate without using this step, as pointed out above. Unfortunately, inosine is also degraded to uric acid which creates a limitation for its use in man. In addition to possible synthesis of AMP, adenosine is converted to inosine by adenosine deaminase. Such inosine can then be utilized in the manner described above.

The human erythrocyte is capable of converting hypoxanthine or guanine to IMP or GMP respectively, with PRPP as a cofactor; the enzyme hypoxanthine-guanine phosphoribosyl transferase (HPRT) catalyses these reactions. HPRT is the enzyme that is deficient in the Lesch-Nyhan syndrome, a sex-linked disease characterised by hyperuricaemia, self-mutilation, choreoathetosis, spasticity and mental retardation (Seegmiller et al., 1967). The presence of the enzyme in the erythrocyte makes it a convenient tissue with which to make the diagnosis. Inosinic acid dehydrogenase links inosine metabolism with guanine nucleotide metabolism (Pehlke et al., 1972) and its activity is increased in the Lesch-Nyhan syndrome.

Both guanine kinase and nucleoside diphosphokinase, additional enzymes of guanine nucleotide metabolism, are present in the human erythrocyte (Agarwal et al., 1971). So far, no specific function for GTP and other guanosine nucleotides in the erythrocyte have been identified (Brewer, 1974).

An enzyme called inosine triphosphatase is present in the erythrocyte (Liakopoulo and Alivasatos, 1964), and an autosomally inherited deficiency of the enzyme has been described (Vanderheiden, 1969). In homozygous affected individuals, elevated levels of ITP accumulate in erythrocytes. The functions of ITP and ITPase are unknown (Brewer 1974).

1.9 2,3-BISPHOSPHOGLYCERATE

1.9.1 Introduction

In recent years 2,3-bisphosphoglycerate (2,3-DPG) has been recognised as a regulatory molecule involved in the control of metabolism and other cellular functions. It is widely present in living cells, functioning as a co-factor for the enzyme phosphoglycerate mutase. Human and many other mammalian erythrocytes contain 2,3-DPG in much higher concentrations than are required for phosphoglycerate mutase activity. It is now generally accepted that 2,3-DPG serves as an important allosteric regulator of haemoglobin function

(Duhm and Gerlach, 1974; Duhm, 1975a and b; Chiba and Sasaki, 1978; Sasaki et al., 1982; Black et al., 1985).

2,3-DPG, like cyclic nucleotides, is formed from readily available molecules, but is not itself part of a major metabolic pathway. It is neither a biosynthetic precursor nor an intermediate in energy metabolism. Hence, its concentration can be independently controlled.

In view of its importance, great efforts have been made to clarify the functions and metabolism of 2,3-DPG in human erythrocytes. A large number of papers on these topics have been published, and consequently this review deals with only some of the more pertinent findings and implications, the purpose being to summarise the various aspects of 2,3-DPG metabolism which are related to changes of 2,3-DPG concentrations as well as to the concomitant alterations of the oxygen affinity of haemoglobin.

1.9.2 Historical remarks

In 1925 Greenwald reported that a major component of organic phosphates in human, pig and dog erythrocytes was 2,3-DPG. Subsequently, Rapaport and Guest (1941) demonstrated that this held for most other mammalian erythrocytes with exceptions such as ruminants. In 1949, Sutherland et al., (1949a and b) found a physiological function of 2,3-DPG: to act as an essential cofactor in the reaction catalysed by phosphoglycerate mutase from animals. The 2,3-DPG level in

many mammalian erythrocytes, however, exceeds by more than two orders of magnitude that needed for maximal activity of phosphoglycerate (Duhm and Gerlach, 1974). This compound can therefore be expected to participate not only in the phosphoglycerate mutase reaction but also in other biochemical events.

In the history of haemoglobin research, data sufficient to explain the physiological significance of these high 2,3-DPG concentrations can be found. In 1909, Barcroft and coworkers (Barcroft and Camis, 1909; Barcroft and Roberts, 1909) reported that the oxygen affinity of haemoglobin increased when erythrocytes were diluted by haemolysis, and as early as 1921 Adair et al. speculated on the presence of a third substance involved in the formation of the oxygen-haemoglobin complex. Hill and Wolvekamp (1936) demonstrated that the dialysable material from fresh erythrocytes decreased the affinity of haemoglobin for oxygen. It is surprising that after Greenwald's discovery of 2,3-DPG in erythrocytes in 1925 more than 40 years passed before its effect on the oxygen affinity of haemoglobin was examined. Almost simultaneously, Benesch and Benesch (1967) and Chanutin and Curnish (1967) reported that 2,3-DPG lowered the oxygen affinity of haemoglobin. Their findings, together with evidence in subsequent papers, have established that 2,3-DPG functions as an effector to facilitate the release of oxygen from oxygenated haemoglobin to tissues.

1.9.3 Enzymes involved in the metabolism of 2,3-DPG

Simplified pathways of glycolysis and of phosphoglycerate metabolism are shown in Figure 1.4. A more detailed pathway for phosphoglycerate metabolism is given in Figure 1.5. 1,3-DPG, which is an intermediate of glycolysis, can be converted to 3-phosphoglycerate (3-PG) in two ways. In most tissues it is converted to 3-PG directly in the phosphoglycerate kinase reaction. Only in the erythrocyte is 1,3-DPG metabolised to a significant extent by 1,3-DPG mutase; 2,3-DPG is then metabolised to 3-PG and inorganic phosphate (Pi) by 2,3-DPG phosphatase. One important difference of the two pathways of 1,3-DPG metabolism concerns the fact that the phosphoglycerate kinase (PGK) reaction yields one high energy phosphate bond in the form of ATP, whereas in the 2,3-DPG bypass, the chemical energy of the acyl phosphate group of 1,3-DPG is dissipated.

The enzymatic reactions implicated in the synthesis and breakdown of 2,3-DPG (Figure 1.5) were first described by Rapoport and Luebering (1950 and 1951). Originally they were attributed to different enzymes:

A) diphosphoglycerate mutase (EC 5.4.2.4), glycerate 3-P + glycerate 1,3-P_2 ⟶ glycerate 2,3-P_2 + glycerate 3-P;

and

B) diphosphoglycerate phosphatase (EC 3.1.3.13), glycerate 2,3-P_2 ⟶ glycerate 3-P + Pi.

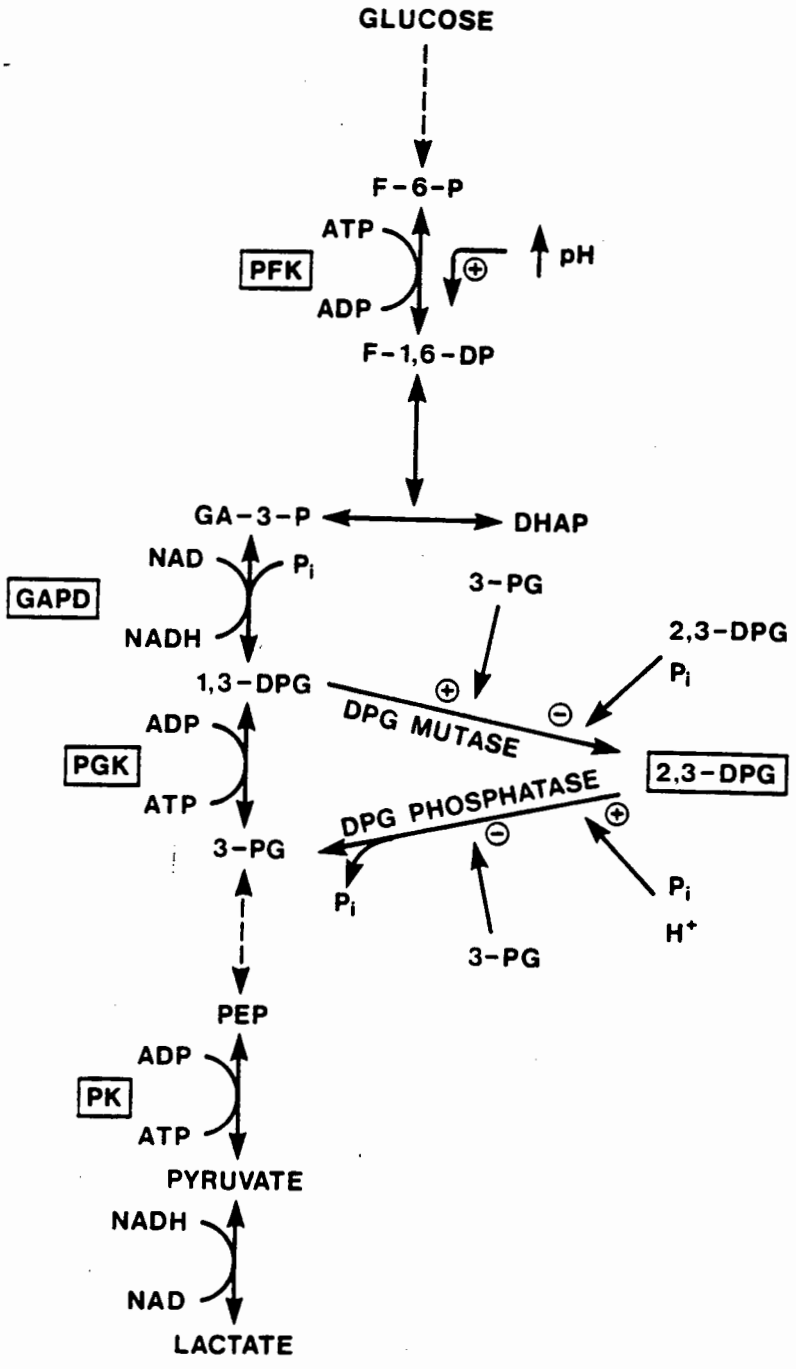
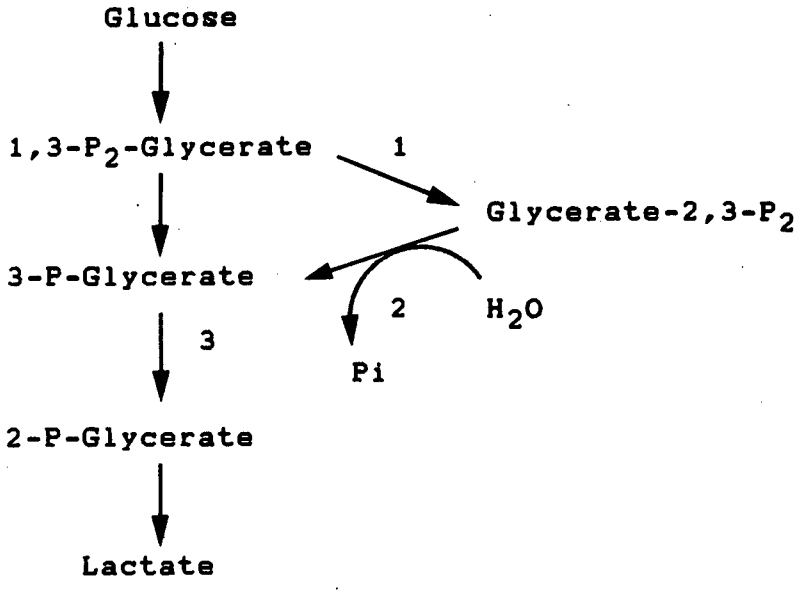


Figure 1.4. Pathways of glycolysis and of phosphoglycerate metabolism in human erythrocytes and factors influencing the formation and decomposition of 2,3-DPG (Duhm and Gerlach, 1974). See Fig. 1.1 for abbreviations.

Figure 1.5. Pathway of 2,3-DPG metabolism.



1) 2,3-DPG synthase bisphosphoglycerate mutase, EC 5.4.2.4).

glycerate 3-P + glycerate 1,3-P_2 ⟶

glycerate 2,3-P_2 + glycerate 3-P.

2) 2,3-DPG phosphatase bisphosphoglycerate phosphatase, EC 3.1.3.13).

glycerate 2,3-P_2 ⟶ glycerate 3-P + Pi

3) 2,3-DPG-dependent phosphoglycerate mutase (EC 5.4.2.1)

glycerate 3-P $\xrightarrow{2,3\text{-DPG}}$ glycerate 2-P.

However, it was found (Chiba and Sasaki, 1978; Sasaki et al., 1982; Rose, 1980) that in mammalian erythrocytes enzymes exist which possess both diphosphoglycerate mutase (2,3-DPG synthase) and diphosphoglycerate phosphatase (2,3-DPG phosphatase) activities. Eight enzyme forms which can be involved in the metabolism of 2,3-DPG have been isolated from tissues in the pig (Carreras et al., 1986). Six of them are multifunctional enzymes which show 2,3-bisphosphoglycerate synthase, 2,3-bisphosphoglycerate phosphatase and phosphoglycerate mutase activities. They differ in their functional and physical properties. The two other enzymes isolated from mammalian tissues possess only 2,3-DPG phosphatase activity.

Since distribution of the eight enzymatic forms varies in different tissues, their relative contribution to the synthesis and degradation of 2,3-DPG differs with the tissue (Carreras et al., 1986). In human erythrocytes and in the erythrocytes of other mammals with high 2,3-DPG levels, both synthesis and breakdown of 2,3-DPG are mainly controlled by 2,3-DPG synthase-phosphatase. In contrast, in other cells the synthesis and degradation of 2,3-DPG involves different enzymes (Carreras et al., 1986). Cat erythrocytes possess low levels of 2,3-DPG. In these cells, 2,3-DPG synthase-phosphatase is present at very low concentration and phosphoglycerate mutase is the enzyme mainly responsible for both synthesis and breakdown of 2,3-DPG (Pons et al., 1985).

1.9.4 Factors controlling 2,3-DPG levels in erythrocytes

The actual size of the 2,3-DPG pool in erythrocytes is determined by the dynamic equilibrium between synthesis and degradation. Judging from the 2,3-DPG mutase activity in haemolysates, human erythrocytes can maximally synthesise about 200 µmoles of 2,3-DPG per hour per millilitre of packed cells (Sasaki et al., 1975). 2,3-DPG phosphatase has the lowest activity of all the enzymes connected with glycolysis in human erythrocytes, but it is activated by several anions. Rose and Liebowitz (1970a) reported that the velocity of the enzyme activity in the absence of activators was 0.06 µmole per hour per millilitre of packed cells and the maximal activity in the presence of physiological activators was 0.84 µmole per hour per millilitre of packed cells.

Human erythrocytes consume 2-3 µmoles of glucose per hour per millilitre of packed cells at 37°C and pH 7.4. The share of the 2,3-DPG bypass has been estimated to be 15-25% of the overall flux through glycolysis (Duhm et al., 1969; Hamasaki and Minakami, 1972; Rapoport et al., 1977; Reich, 1968; Rose and Liebowitz, 1970a and b). From these values, the turnover rate of 2,3-DPG can be calculated to be 0.3 - 0.75 µmoles/hr/ml packed erythrocytes. The DPG mutase activity in erythrocytes therefore appears to be partially latent, whereas 2,3-DPG phosphatase works almost maximally. The kinetic data available for considering regulatory mechanisms of DPG mutase and 2,3-DPG phosphatase are in large part due

to the efforts of Rose and co-workers (Rose, 1968 and 1973; Rose and Liebowitz, 1970a; Rose and Dube, 1976).

1.9.4.1 2,3-DPG synthesis

The rate of 2,3-DPG synthesis in erythrocytes first of all depends on the concentration of 1,3-DPG, since under normal conditions the concentration of 1,3-DPG is smaller than the Km value of DPG mutase (0.5 micromolar) (Rose, 1968). Additional factors directly involved in the regulation of 2,3-DPG synthesis are the concentration of 2,3-DPG itself and the concentrations of 3-PG and inorganic phosphate (Rose, 1968 and 1970; Minakami and Yoshikawa, 1965 and 1966a and b; Tuboi and Fukunaga, 1965).

The concentration of 1,3-DPG is governed by the concerted action of several glycolytic enzymes (Figure 1.4:

1. Phosphofructokinase (PFK). This enzyme is known to control the main rate-limiting step of glycolysis. The activation of PFK, which can be induced by many factors (e.g., elevation of pH, increase in Pi, ADP or AMP, decrease of ATP) (Passaneau and Lowry, 1964; Rapoport, 1968) must therefore lead to increased formation of 1,3-DPG.

2. Glyceraldehyde-3-phosphate dehydrogenase (GAPD). This enzyme can become rate-limiting under certain conditions, e.g., at elevated pH and a diminished ratio of NAD: NADH (Rapoport, 1968). Pyruvate and methylene blue (oxidised) are able to induce an increase in the NAD:NADH ratio and thus

promote an increase in 1,3-DPG levels (Rose and Warms, 1970). An increase in Pi will also result in increased formation of 1,3-DPG.

3. Phosphoglycerate kinase (PGK). Inhibition of this enzyme causes an accumulation of 1,3-DPG. Since ATP, ADP and 3-PG are near to thermodynamic equilibrium with 1,3-DPG concentration, changes in each of these compounds must be reflected in changes in 1,3-DPG levels (Duhm and Gerlach, 1974).

4. Pyruvate kinase (PK). A decrease in the activity of this enzyme can result in a fall in the ratio of ATP:ADP and an elevation of phosphoenol pyruvate (PEP), 2-PG and 3-PG levels, since the enolase and the phospholgycerate mutase reactions are close to thermodynamic equilibrium (Minakami and Yoshikawa, 1966a and b). Because such alterations concern the reactants of the PGK reaction, 1,3-DPG levels must increase after inhibition of pyruvate kinase (Duhm and Gerlach, 1974).

5. 2,3-DPG mutase. The rate of 2,3-DPG synthesis depends not only on 1,3-DPG concentration but also on the level of 2,3-DPG. DPG mutase is strongly inhibited by its product 2,3-DPG (Ki = 0.8 µM) (Rapoport and Luebering, 1952; Rose, 1968). At physiological levels of 2,3-DPG, the inhibition amounts to about 99% (Gerlach and Duhm, 1972), accordingly, DPG mutase is working at only 1% of its potential capacity

under physiological conditions. In principle therefore, a decrease in 2,3-DPG should cause a relief of the product inhibition followed by increased synthesis of 2,3-DPG.

Compared to the importance of 1,3-DPG and 2,3-DPG in the regulation of 2,3-DPG synthesis, the influence of 3-PG and inorganic phosphate on DPG mutase activity is of minor importance (Rose, 1968 and 1970). This is due to the fact that under most conditions, changes in the concentrations of these compounds are not great enough to induce significant alterations in the activity of DPG mutase. An exception would be in tissue ischaemia where Pi concentrations may rise 20 to 30 fold (Radda, 1983; Radda et al., 1983).

1.9.4.2 2,3-DPG degradation

As pointed out, the formation of 2,3-DPG depends strongly on the concentration of 1,3-DPG, the substrate of DPG mutase. In contrast, there is no such relationship in human erythrocytes between substrate (2,3-DPG) concentration and the rate of its degradation because of the low Km value of 2,3-DPG phosphatase (Km = 1 μM) (Rose and Liebowitz, 1970a and b). It thus appears that changes in the rate of 2,3-DPG decomposition can only result from alterations in the activity of 2,3-DPG phosphatase.

Hydrogen ion concentration appears to be of particular importance in influencing the activity of 2,3-DPG phosphatase (Hashimoto et al., 1961; Grisolia et al., 1968; Rose, 1969;

Sauer et al., 1968; de Verdier and Groth, 1973). The pH optimum of this enzyme is 6.5 (de Verdier, 1964), thus a decrease of the intracellular pH from its normal value of 7.2 causes an acceleration of 2,3-DPG degradation. Inorganic phosphate is also known to activate 2,3-DPG phosphatase (Rose, 1970). We shall see in Chapters 2 and 3 that Pi levels in the erythrocyte increase with a drop in pH.

Several investigators have studied the effects of inorganic sulphur compounds on the activity of 2,3-DPG phosphatase; some have been found to activate it and others to inhibit it (Duhm and Gerlach, 1974). The inhibitory action of sulphate may be of special significance for 2,3-DPG metabolism in erythrocytes of uraemic patients, since it is conceivable that the increased levels of red cell 2,3-DPG found in this condition may at least be partly related to the well-known elevation of plasma sulphate found in uraemic patients (Duhm and Gerlach, 1974).

It must be emphasised that the activity of 2,3-DPG phosphatase is fairly low compared with the activity of DPG mutase (in human erythrocytes 0.01 and 3 to 5 μmoles/gm/min respectively) (Duhm and Gerlach, 1974; Rose, 1968; Rapoport, 1968).

1.9.4.3 Pathophysiological conditions which affect 2,3-DPG concentration in erythrocytes

The concentration of 2,3-DPG in erythrocytes changes in vivo in a number of physiological and pathological conditions (Table 1.1). Most of these changes and the resulting displacements of the oxyhaemoglobin dissociation curve constitute one among several adaptive processes which together are involved in the improvement of the oxygen supply to tissues (Mairbaurl et al., 1986; Duhm and Gerlach, 1974).

Studies concerning the influence of exercise and training on 2,3-DPG content of erythrocytes have given divergent results. Although some authors have reported an increase in 2,3-DPG concentration, others could not confirm these findings (Remes et al., 1979). Most kinds of acute and chronic hypoxia (anaemia, abnormalities in haemoglobin affinity for oxygen, pulmonary diseases, reduction in alveolar pO_2), as well as respiratory or metabolic alkalosis, produce an increase in erythrocyte 2,3-DPG. This increase can be attributed to a decrease in the free 2,3-DPG concentration due to greater binding of the metabolite to deoxyhaemoglobin, and to an increase in cellular pH (Benesch and Benesch, 1967 and 1974; Benesch et al., 1968a and b). The decrease in free 2,3-DPG would enhance its synthesis by relieving 2,3-DPG synthase from product inhibition. The increase in pH would elevate 2,3-DPG levels by increasing glycerate 1,3-P_2 concentration by stimulating glycolysis, and by changing the catalytic properties of 2,3-DPG synthase-phosphatase, stimulating the

TABLE 1.1

Factors and conditions which affect the level of 2,3-DPG in erythrocytes

Condition	Effect
Exercise	I
Hypoxia	I
Respiratory and metabolic alkalosis	I
Abnormalities of glycolytic enzymes	I/D
Hepatic cirrhosis	I
Kidney diseases	I
Neoplastic diseases	I
Hyperthyroidism	I
Androgens	I

I : increases

D : decreases

synthase and reducing the phosphatase activity (Duhm and Gerlach, 1974; Brewer and Eaton, 1971; Finch and Lenfant, 1972). Erythrocyte levels of 2,3-DPG rise in patients suffering from hepatic cirrhosis, uraemia and other kidney diseases, neoplastic and toxic diseases (Duhm and Gerlach, 1974; Chiba and Sasaki, 1978; De la Morena, 1979 and 1984). The factors involved in the changes of 2,3-DPG in these diseases are not well understood.

Congenital abnormalities in erythrocyte glycolytic enzymes can alter 2,3-DPG concentration. Rate-limiting enzymatic abnormalities of the glycolytic pathway before the stage of glycerate 1,3-P_2 formation reduce the amount of 2,3-DPG and therefore increase oxygen affinity. Alterations located after that stage may either increase or decrease 2,3-DPG concentration (Finch and Lenfant, 1972).

It has been found that erythrocyte 2,3-DPG levels increase in hyperthyroidism and after incubation of normal blood in the presence of thyroid hormones. A correlation has been shown between reduction in aldosterone excretion in urine and a decrease in erythrocyte 2,3-DPG. It has also been reported that androgens increase levels of 2,3-DPG in erythrocytes. The underlying mechanisms of these hormonal effects remain unclear (Duhm and Gerlach, 1974; Chiba and Sasaki, 1978; Finch and Lenfant, 1972).

1.9.4.4 Deficiency of the enzymes implicated in 2,3-DPG metabolism

Deficiencies of two of the multifunctional enzymes involved in 2,3-DPG metabolism, erythrocyte 2,3-DPG synthase-phosphatase and muscle-type phosphoglycerate mutase, have been reported. In 1978, Rosa et al. described a case of an inherited and complete deficiency of 2,3-DPG synthase-phosphatase in human erythrocytes. The level of 2,3-DPG was found to be below 3% of normal values. Accordingly, the oxygen affinity of the erythrocytes was increased, although the oxygen saturation curve of the haemolysate free of 2,3-DPG was normal. 2,3-DPG synthase and 2,3-DPG phosphatase activities were undetectable, and phosphoglycerate mutase activity was about half of the mean normal value. All other erythrocyte enzyme activities were normal. It was suggested that the small amounts of 2,3-DPG present could have been synthesized by phosphoglycerate mutase (Rosa et al., 1978), as has been shown to be the case in certain mammalian erythrocytes with low levels of 2,3-DPG (Carreras et al., 1983; Pons et al., 1985).

Muscle type M phospholgycerate mutase deficiency has recently been described in a patient with intolerance for strenuous exercise. In agreement with the tissue distribution of the type M (muscle) isoenzyme, the levels of phosphoglycerate mutase activity were found to be markedly decreased in skeletal muscle but only slightly reduced in erythrocytes, leukocytes and cultured muscle. The electrophoretic pattern

showed that the residual phosphoglycerate mutase activity in the patient's muscle corresponded to the brain-type isoenzyme, normally present in very small quantities in adult muscle (Di Mauro et al., 1981 and 1982).

1.9.5 Functions of 2,3-DPG

Most mammalian erythrocytes possess 2,3-DPG in high concentration (4-13 mmol/l; humans, 5 mmol/l). Exceptions are the erythrocytes of some carnivores and artiodactyles which contain low levels (0.1 - 1 mmol/l) (Bunn et al., 1974; Vanderheiden, 1961). 2,3-DPG influences many biochemical events in the erythrocytes of humans and these influences are summarized in Table 1.2.

1.9.5.1 Effect on oxygen affinity of haemoglobin

The major role of 2,3-DPG in erythrocytes is to act as an allosteric modulator of the affinity of haemoglobin for oxygen. Oxygenation of haemoglobin leads to a conformational change in its quaternary structure (Wyman, 1964). According to the X-ray crystallographic studies of Perutz et al. (1970) the transition from the deoxy- to the oxy- conformation is triggered by changes in the diameter of the iron atoms (upon oxygenation) in the haem groups which induce alterations of the tertiary structure of the subunits.

It is now well established that 2,3-DPG is preferentially bound to deoxyhaemoglobin, whereas the oxy- conformation

TABLE 1.2

Possible functions of 2,3-DPG in erythrocytes.

Ref. Chiba and Sasaki, 1978.

Primary effect	Secondary effects
Binding to HB	. Decrease in O_2 affinity of Hb
	. Change in Bohr effect
	. Decrease in CO_2 binding to Hb
	. Stimulation of gelation of deoxygenated sickle cell Hb
	. Change in concentration of free ATP, 2,3-DPG and Mg^{2+}
Participation in Donnan distribution	. Change in erythrocyte pH
	. Change in O_2 affinity of Hb
	. Changes in activities of pH-dependent enzymes
Interaction with enzymes or chelation of Mg^{2+}	. Change in activity of metabolic pathways
Unknown effect	. Inhibition of platelet aggregation

exhibits only a low affinity for 2,3-DPG because of a different steric arrangement of the binding sites (Benesch et al., 1968a and b; Perutz, 1970). Due to the binding of 2,3-DPG, the deoxy- conformation is stabilized and the allosteric equilibrium between the oxy- and deoxy-conformation is thereby shifted favouring the deoxy-structure. Consequently the 2,3-DPG induced stabilization of the deoxy- conformation must lead to a decrease in the oxygen affinity of haemoglobin thus facilitating the supply of oxygen to tissues (Benesch and Benesch, 1967 and 1974; Benesch et al., 1968a and b). From these findings it appears that four phenomena are causally related to each other: 1) the conformational change of the haemoglobin tetramer during oxygenation, 2) the difference in the oxygen affinities of the two quaternary conformations, 3) the preferential binding of 2,3-DPG to the deoxy- conformation, and 4) the reduction of the oxygen affinity of haemoglobin by 2,3-DPG.

Studies have shown that only one molecule of 2,3-DPG can be bound to one deoxyhaemoglobin tetramer and that only isolated beta- chains, but not isolated alpha- chains, react with 2,3-DPG (Benesch et al., 1968a and 1968b). This provided evidence for the binding of 2,3-DPG to the beta-chains along the "dyad axis of symmetry" of the haemoglobin tetramer. It was subsequently demonstrated that 2,3-DPG binds to histidyl groups 143 of the beta- chains and to the two N-terminal valines of the beta- chains, all of which are directed into a central cavity of the tetramer (Duhm and Gerlach, 1974). It

has furthermore been demonstrated that CO_2 reduces the binding of 2,3-DPG and its effect on the oxygen affinity of haemoglobin by forming carbamate groups with the N-terminal values of the beta- and alpha- chains (Bauer and Schroder, 1972; Duhm and Gerlach, 1974).

According to Perutz (1970) the 2,3-DPG molecule fits into the central cavity of the deoxyhaemoglobin tetramer in such a way that the anionic groups of 2,3-DPG are within hydrogen-bonding distance of the histidyl groups 143 and of the two N-terminal amino groups of the two beta- chains. The amino group of lysine 82 in one beta- chain can also react with 2,3-DPG. Upon oxygenation, conformational changes are induced which result in the central cavity becoming narrower. Furthermore, the distance between the two N-terminal amino groups of the beta- chains increases and therefore the affinity of binding of the 2,3-DPG molecule to both of them decreases. Thus 2,3-DPG is released from its binding sites during oxygenation of deoxyhaemoglobin. Conversely, the preferential binding of 2,3-DPG with deoxyhaemoglobin displaces the $HbO_2 \longleftrightarrow Hb$ equilibrium to the deoxygenated form.

2,3-DPG is not the only compound which reduces the oxygen affinity of haemoglobin by differential binding to the oxy- and deoxy- conformations. Among these compounds ATP (Benesch et al., 1986) and phytic acid are of physiological importance, whereas GTP, pyridoxal-5-phosphate, hexameta-

phosphate and a fluorescent analogue of 2,3-DPG as well as certain spin labels are of theoretical interest (Duhm and Gerlach, 1974). It has been found that those species with high levels of erythrocyte 2,3-DPG possess haemoglobins of intrinsically high oxygen affinity. In contrast, those species with low levels of erythrocyte 2,3-DPG possess haemoglobins with relatively low oxygen affinity and which are unresponsive to 2,3-DPG. Thus, the level of 2,3-DPG in each species is appropriate for the functional capability of its haemoglobin (Bunn et al., 1974). During embryonic and foetal development a shift in the type of synthesized haemoglobin occurs, haemoglobins with different oxygen affinity appearing. The concentration of 2,3-DPG in erythrocytes during development varies in the appropriate way to keep the oxygen affinity of the foetal red cells higher than that of the maternal red cells, and to ensure oxygen transport across the placenta (Chiba and Sasaki, 1978; Sasaki et al., 1982).

1.9.5.2 Control of Glycolysis

The rate of glycolysis in human erythrocytes is about one-fifth the maximal activity of hexokinase, which has the lowest activity of all the glycolytic enzymes (Jacobasch et al., 1974; Bishop, 1964). Thus the glycolytic enzymes are not saturated by their substrates, whose concentrations are regulated mainly by three enzymes, hexokinase, phospho-fructokinase and pyruvate kinase. These key enzymes are primarily responsible for the regulation of glycolysis in

human erythrocytes because the reactions they catalyze are far from thermodynamic equilibrium (Minakami and Yoshikawa, 1966a). The activities of these enzymes are controlled by substrates, cofactors and effectors, including 2,3-DPG.

Duhm (1975b) prepared erythrocytes containing five times the normal amount of 2,3-DPG and found that the glucose consumption and lactate production rates in such cells were reduced by 70% and 40% respectively, compared to those of normal cells. About 50% of the inhibition of glycolysis in "high 2,3-DPG" cells was attributed to direct inhibitory effects of 2,3-DPG on hexokinase and pyruvate kinase, and the remaining 50% to a reduction in phosphofructokinase activity caused by a 2,3-DPG-induced decrease in intracellular pH.

1.9.5.3 Other functions

In addition to decreasing the affinity of haemoglobin for oxygen and its role in the control of glycolysis, several other functions have been postulated for 2,3-DPG in mammalian erythrocytes. By binding to haemoglobin, 2,3-DPG has several actions: it influences the effect of pH on the oxygenation curve of haemoglobin (the Bohr effect), decreases CO_2 binding to haemoglobin, has a biphasic influence on the tetramer-dimer equilibrium of carbon monoxide haemoglobin (Gray and Dean, 1987), changes the concentration of free ATP and Mg^{2+}, and stimulates methaemoglobin reduction by ascorbate (Duhm and Gerlach, 1974; Chiba and Sasaki, 1978). The role that 2,3-DPG has to play in erythrocyte purine

metabolism is discussed in Chapter 2. 2,3-DPG can develop other functions not related to haemoglobin binding. By interacting with cytoskeleton proteins, it may influence the deformability of the red cell membrane and the lateral mobility of integral membrane proteins (Sheetz and Casaly, 1981; Mentzer et al., 1987). By interacting with the transport system, it may inhibit the transport of phosphoenolpyruvate across the erythrocyte membrane (Hamasaki, 1984). Through its participation in the establishment of a Donnan distribution of hydrogen ions between erythrocytes and plasma, 2,3-DPG can affect the oxygen affinity of haemoglobin as well as the activity of several enzymes (Chiba and Sasaki, 1978). 2,3-DPG may also affect enzyme activity by either chelating Mg^{2+} or interacting directly with the enzymes (Chiba and Sasaki, 1978). It has been shown that at physiological concentrations 2,3-DPG inhibits several enzymes involved in carbohydrate and adenine nucleotide metabolism (Table 1.3), although it has not been shown that all these effects are important in the regulation of the metabolic pathways in erythroid cells (Chiba and Sasaki, 1978).

It has recently been shown that, by binding at the substrate binding site, 2,3-DPG can act both as an activator and an inhibitor of casein kinase II from rabbit reticulocytes (Hathaway and Traugh, 1984a and b). On this basis it has been suggested that intracellular fluctuations in the levels of 2,3-DPG could regulate casein kinase II activity in

TABLE 1.3

Effects of 2,3-DPG on Enzyme Activities

Enzyme	Source	Effect
Hexokinase	E	I
Phosphofructokinase	E,M,B	I
Pyruvate kinase	E	I
Phosphoglycerate kinase	E	I
2,3-DPG synthase	E,M	I
Glyceraldehyde 3-P dehydrogenase	E,M	I
Aldolase	E	I
Phosphoglucomutase	E,M,L	I
G-1,6-P_2 synthase	M,B	I
6-Phosphogluconate dehydrogenase	E	I
Transaldolase	E	I
Transketolase	E	I
AMP deaminase	E,M	I
HPRT	E	I
APRT	E	I
PRPP synthetase	E	I
Purine 5'-nucleotidase	E	A
Casein kinase II	R	I/A

Key: E: erythrocytes; M: skeletal muscle; H: heart; B: brain; L: liver; R: rabbit erythrocytes. Effects: A: activation; I: inhibition.

erythroid cells and thus influence protein synthesis and other cellular functions (Hathaway and Traugh, 1984a). Inhibition of protein synthesis by 2,3-DPG in rabbit reticulocyte lysates has already been reported (Narita et al., 1979).

In cells other than erythrocytes the only known function for 2,3-DPG is to act as an essential cofactor in the interconversion of glycerate-3-P and glycerate-2-P catalysed by the 2,3-DPG dependent phosphoglycerate mutase (EC 5.4.2.1) (Grisolia, 1968). The physiological significance of the inhibitory effect of 2,3-DPG on several enzymes demonstrated in vitro is uncertain, because there is not sufficient information about the concentration of 2,3-DPG in tissues. Due to blood contamination, the concentration of 2,3-DPG in mammalian tissues has not been estimated accurately (Chiba and Sasaki, 1978). In rat tissues Carreras et al. (1986) have detected levels ranging from 0.25 nmol/g wet tissue in brain to 41 nmol/g in liver.

CHAPTER 2

AN OXYPURINE CYCLE IN HUMAN ERYTHROCYTES REGULATED BY pH, INORGANIC PHOSPHATE, AND OXYGEN

2.1 INTRODUCTION

De novo purine synthesis does not take place in mature erythrocytes (Lowy et al., 1962), whereas formation of purine nucleotides by salvage pathways and release as purine bases has long been known to occur (Mager et al., 1966; Hershko et al., 1967; Hershko et al., 1969). It is sometimes stated that this may represent a role for the erythrocyte in the transport of purines from tissues with a purine surplus to tissues with a purine requirement (Henderson and Le Page, 1959; Lowy and Lerner, 1974).

Human erythrocytes can take up and convert to the corresponding nucleotide a number of purines including adenine, adenosine, hypoxanthine and guanine (Mager et al., 1966; Gutenson, 1975). Mager et al. (1966) have pointed out that unlike other purine base substrates, which contribute to di- and triphosphate nucleotide pools, anabolism of hypoxanthine ceases at inosine 5'-monophosphate (IMP). Hypoxanthine is further distinguished by its particularly rapid rate of clearance from erythrocytes as well as the negligible size of its normal intracellular pool (Bishop et al., 1959). This distinctive pattern of metabolism of hypoxanthine is consistent with its role both

as an end product of purine catabolism in the human erythrocyte (Bishop et al., 1959; Hershko et al., 1963; Schraufstatter and Born, 1981) and as a mediator in the postulated two-way traffic of purines between the liver and peripheral tissues (at least 95% of the hypoxanthine pool is recycled), in which the erythrocyte may serve as the transporting vehicle (Henderson and Le Page, 1959; Zeitlin-Beck et al., 1964; Soshani et al., 1965).

Hypoxanthine is transported rapidly across the red cell membrane by both saturable and non-saturable carrier systems (Lassen, 1967). In this respect erythrocytes differ from cultured nucleated cells which possess only the saturable system (Marz et al., 1979). Equilibrium is reached within a minute even at millimolar concentrations of hypoxanthine in the medium (Lassen, 1967). Initially it was reported that in erythrocytes from fresh heparinised blood little or no conversion of hypoxanthine to IMP occurred (Lowy et al., 1962; Bishop et al., 1959). Later it was found that whereas this appeared to be generally correct for freshly drawn blood, where the physiological concentration of Pi in plasma is around 1 mM, if the erythrocytes were incubated in medium containing higher levels of inorganic phosphate (Mager et al., 1966; Hershko et al., 1969; Gutenson, 1975; Giacomello and Salerno, 1979) there was conversion of hypoxanthine to IMP with an optimum external inorganic phosphate concentration of around 20 to 30 mM (Mager et al., 1966; Gutenson, 1975).

Hershko et al. (1969) have demonstrated that synthesis of 5-phosphoribosyl-1-pyrophosphate (PRPP) in erythrocytes follows sigmoid kinetics with respect to inorganic phosphate. At neutral pH the rate of synthesis was negligible at external inorganic phosphate concentrations below 5 mM, but showed progressive enhancement with increasing external inorganic phosphate, reaching levels approaching 100 nanomoles/ml cells/hr at external inorganic phosphate concentrations of 30 mM. It is now generally accepted that the limiting factor in the ability of erythrocytes to synthesize IMP from hypoxanthine is the availability of PRPP (Hershko et al., 1969; Henderson and Le Page, 1959; Giacomello and Salerno, 1979). PRPP synthetase is inhibited by ADP, GDP and 2,3-bisphosphoglycerate (2,3-DPG) and stimulated by inorganic phosphate (Fox and Kelley, 1971a and b). Even under experimental conditions of high external inorganic phosphate concentrations the synthesis of PRPP amounts to only 1% of the rate observed in equivalent amounts of stroma-free haemolysate (Hershko et al., 1969) and has been attributed to allosteric inhibition by ADP and 2,3-DPG (Hershko et al., 1969; Sciaky et al., 1974).

Little quantitative information has been published on the effect of pH on IMP formation or degradation in erythrocytes. Salerno et al. (1986) have reported that incorporation of hypoxanthine into IMP increased with decreasing pH whereas Schraufstatter and Born (1981) reported that this was independent of pH. The latter did, however, report that

hypoxanthine and some inosine were released from erythrocytes labelled with ^{3}H-Adenine or ^{3}H-Adenosine at an accelerated rate at alkaline pH values. Bontemps et al. (1986) reported that alkalinisation of the incubation medium resulted in a fifteen-fold increase in the rate of production of hypoxanthine from erythrocytes. Uraemic subjects (who are well known to be acidotic and to have raised serum phosphate levels) were noted to have raised erythrocyte IMP levels by Mansell et al (1981) and Angle et al. (1985).

Similarly, there are few reports on the effect of oxygen partial pressure on hypoxanthine uptake and release. Salerno et al. (1986) have recently reported that oxygen can lower the rate of IMP synthesis in human erythrocytes. Henderson and Le Page (1959) and Hershko et al. (1967) reported that loss of adenine nucleotide purine from erythrocytes was greater under aerobic than anaerobic conditions. However, Schraufstatter and Born (1981) found no difference under their conditions at pH 7.4.

Here, the uptake and release of hypoxanthine under conditions of varying inorganic phosphate concentrations, pH and pO_2 have been studied. It has been shown that uptake and conversion of hypoxanthine to IMP is enhanced by increasing inorganic phosphate, and decreasing pH and pO_2. It has also been shown that release of IMP as hypoxanthine is favoured at low inorganic phosphate concentrations, high pH and high pO_2.

2.2 MATERIALS AND METHODS

2.2.1 Metabolism of oxypurines by erythrocytes

Fresh heparinised human blood obtained by venipuncture was centrifuged at 4°C for 12 minutes at 1700 g and the plasma, buffy coat and top 1/5th of red cells aspirated and discarded to remove nucleated species. The remaining cells were then washed twice with 3 volumes of ice-cold 0.9% saline. The washed erythrocytes were then incubated in medium consisting of HEPES (50 mM), glucose (10 mM), Na_2HPO_4 (10 mM) and NaCl (75 mM) at a packed cell to medium ratio of 1:2 (v/v). pH was adjusted by the addition of 1 M HCl or 1 M NaOH. In some experiments the medium contained in addition ^{14}C-hypoxanthine (100 µM, 1.2 Ci/mol) (Amersham, Buckinghamshire, England) or ^{32}Pi (5 mCi/mol) (Amersham, Buckinghamshire, England). To quantitate labelled purine species, an aliquot of suspension, typically 0.5 ml, was briefly centrifuged at 10000 g through dibutylphthalate (Merck, Schuchardt, Germany), as described by Wohlhueter et al. (1978) in a 1.5 ml microfuge tube, and after aspiration, the supernatant medium deproteinized with an equal volume of 0.6 M perchloric acid. The dibutylphthalate was aspirated and the red cell pellet resuspended in an equal volume of isotonic saline, followed by 2 volumes 0.6 M perchloric acid. After centrifugation an aliquot of the clear supernatant was mixed with scintillation fluid and counted by scintillation photometry using an open window setting. From the specific activity, the uptake of purine or phosphate could be

expressed in μM or mM respectively. In some cases aliquots were neutralized with 2.5 M K_2CO_3, and after centrifugation the supernatant (typically 50 μl) was injected onto an anion exchange or reverse phase HPLC column (details of HPLC given below). Eluate fractions were counted and from the position of markers individual labelled purine species were identified and quantitated. In experiments where ^{32}Pi was used, the individual labelled ^{32}P species were separated in a similar manner, using anion exchange HPLC. The Pi concentration of cells and medium was measured by the colorimetric method of Fiske and SubbaRow (1925). The pH of the red cell suspensions was determined to 2 decimal places using an ABL blood gas analyser (Radiometer, Copenhagen, Denmark).

Incubation of red cells in whole blood was performed by addition of Na_2HPO_4, glucose, and ^{14}C-hypoxanthine to freshly drawn heparinized blood to give a final concentration of 10 mM, 10 mM and 100 μM respectively without significant increase in volume. pH was reduced to approximately 7 by equilibration with 20% CO_2 in oxygen. Samples were removed before and after a 90 minute incubation to determine hypoxanthine uptake. Following incubation, erythrocytes were isolated by centrifugation through dibutylphthalate and resuspended in autologous fresh plasma. A baseline sample was removed at this stage. The pH of the suspension was increased by equilibration with air to displace CO_2. Samples of varying pH were removed during this procedure and incubated for 1 hour to determine hypoxanthine release. The

concentration of Pi, total labelled purine, and in some cases individual purines was determined as described above for cells incubated in defined aqueous medium. Blood pH and pO_2 was measured on an ABL blood gas analyser (Radiometer, Copenhagen, Denmark). Plasma lactate was measured on the perchloric acid extracts by monitoring the formation of NADH in the presence of lactate dehydrogenase (Bergmeyer, 1974).

2.2.2 Cell-free preparations (dialysed haemolysate)

Haemolysate was prepared according to Steyn and Harley (1984). Enzyme extracts were prepared from 10 ml of fresh, heparinised human blood. The plasma and buffy coat were removed after centrifugation at 1700 x g for 10 minutes. The red blood cells were washed twice with 2 volumes of normal saline and were lysed by adding 4 volumes of distilled water at 4°C; the stroma was removed by centrifugation. The haemolysate was dialysed for 16 h at 4°C against two changes of 5 litres of 50 mM Tris-HCl, pH 7.8, containing 1 mM dithiothreitol. The dialysed lysate was frozen in aliquots of 500 µl, and stored at -20°C for not longer than 4 weeks, by which time Steyn and Harley (1984) demonstrated that there was a 10% loss of hypoxanthine-guanine phosphoribosyl transferase (HPRT) activity. The thawed lysates were used in the PRPP assay described below.

2.2.3 PRPP assay

The PRPP content of erythrocytes was measured by slight modification of the method of Hershko et al. (1969), in which ^{14}C-IMP formed in the presence of ^{14}C-hypoxanthine and hypoxanthine-guanine phosphoribosyltransferase (HPRT) reflects the PRPP originally present. Typically, 20 µl EDTA (10 mM) was added to 200 µl aliquots of packed red cells in 1,5 ml microfuge tubes which were heated at 100°C for 1 minute. The samples were chilled and mixed with 50 µl of a 10% suspension of activated charcoal. The charcoal adsorbs endogenous nucleotides and together with precipitated protein was deposited by centrifugation at 10000 g. 30 µl of clear supernatant was incubated for 3 hours at 37°C with 20 µl of a combined reagent containing Tris HCl (0.1 M, pH 8.0), $MgCl_2$ (30 mM), ^{14}C-hypoxanthine (300 µM, 5 Ci/mol) and 40% v/v of the dialysed haemolysate from normal erythrocytes as a source of HPRT. 15 µl aliquots of the incubation mixture were spotted in duplicate onto 2 cm diameter discs marked with pencil on polyethyleneimine (PEI) phosphocellulose thin layer plates (Merck, Darmstadt, Germany) and washed exhaustively with distilled water to remove ^{14}C-hypoxanthine. The discs were then dried, cut out and placed in counting vials with 10 ml Instagel (Packard) and counted in a Beckman LS-233 scintillation counter to quantitate the ^{14}C-IMP bound. The assay was standardized using solutions of 0, 50, 100, 150 and 200 µM PRPP in place of sample.

2.2.4 PRPP synthetase assay

A partially purified preparation of PRPP synthetase was prepared following the procedure of Hershko et al. (1969) and Hennessy et al. (1962). All steps were performed at 4°C. 5 ml of fresh washed packed cells were lysed in 15 ml cold EDTA (1 mM) and stirred for 10 minutes with 200 mg activated charcoal. After centrifugation at 3000 g, the supernatant was stirred for 20 minutes with 10 ml of a 10% suspension of DEAE-cellulose prepared according to Hennessy et al. (1962). Following adsorption of the haemolysate the DEAE-cellulose was washed with 3 mM potassium phosphate, pH 7, in a Buchner funnel until the effluent was colourless. The enzyme was eluted by stirring the DEAE-cellulose for 20 minutes in 10 ml 0.5 M KCl containing 1 mM EDTA following which the matrix was deposited by centrifugatioan and the supernatant enzyme solution stabilized by the addition of 100 mg albumin, dialyzed for 4 hours against Tris HCl (10 mM, pH 7.4) and stored in aliquots at -20°C. The assay of PRPP synthetase was performed according to the two-step procedure of Hershko et al. (1969), but with slight modification. In the first step, synthesis of PRPP was allowed to proceed in a reaction mixture containing, in a final volume of 1.10 ml, the following ingredients: 50 mM Tris-HCl (pH as specified), 5mM $MgSO_4$, 2.5 mM reduced glutathione (GSH), 1 mM EDTA, Na_2HPO_4 (concentration and pH as specified), 0.1 mM ATP, 1 mM Ribose-5-phosphate (R-5-P), 0.1 mM ATP, 1 mM phosphoenol pyruvate, 10 µl pyruvate kinase (Boehringer) and 0.1 ml of the DEAE-cellulose-treated enzyme preparation described above. The

reaction mixture was then incubated at 37°C. At 10 minute intervals, from 0 to 40 minutes, 200 µl aliquots were removed and the reaction terminated by immersing the tubes in boiling water for 1 minute. The tubes were then immediately chilled on ice, and 100 µl of a freshly mixed 10% activated charcoal solution added to adsorb ATP and other nucleotides. The charcoal was deposited by centrifugation and aliquots of the clear supernatant were used in the second step of the assay in which the amount of PRPP formed was determined as described above.

2.2.5 High Pressure Liquid Chromatography (HPLC)

The separation of oxypurine metabolites was effected by HPLC (Spectra Physics Model 3500 B) of neutralized perchloric acid extracts of erythrocytes using both anion exchange and reverse phase columns. Anion exchange chromatography was performed on a Hichrom APS HVP-2511 column (Hichrom, Reading, England), with a linear buffer gradient developed over 10 minutes from 5 mM KH_2PO_4 (BDH, Aristar grade) pH 2.50, to 500 mM KH_2PO_4 plus 912 mM KCl, pH 3.8 at a flow rate of 1.2 ml/min. Depending on the retention times of the metabolites being studied, thirty or sixty second fractions of the effluent were collected, mixed with scintillation fluid (HIONIC-FLUOR, United Technologies Packard, Downers Grove, Illinois, USA) and counted in a Beckman LS 233 liquid scintillation system.

Reverse phase chromatography was performed on a Hichrom S50 DS-3571 column (Hichrom, Reading, England), using 5% methanol in 1 mM KH_2PO_4 (v/v) as an isocratic buffer at a flow rate of 1.6 ml/min. Fractions were collected and counted as described above. In all cases the absorbance of the effluent was monitored at 260 nm.

2.2.6 2,3-Bisphosphoglyceric Acid (2,3-DPG) assay

2,3-DPG was quantitated using a commercial kit (Sigma Chemical Company, St Louis, USA, catalog number 665). The principle of the test is as follows: 2,3-DPG is enzymatically hydrolysed to 3-PGA and Pi. The enzyme which catalyses this reaction is present in purified preparations of phosphoglycerate mutase (PGM) and is termed 2,3-DPG phosphatase. 2-Phosphoglycolic acid is needed as an activator for this reaction (Rose, 1970; Rose and Liebowitz, 1970b). It is not yet certain whether this reaction is caused by a contaminating enzyme in the PGM or is the result of a secondary function of PGM (Lowry et al., 1964). The reaction is allowed to go to completion and the resulting Pi is then colourimetrically determined by the method of Fiske and SubbaRow (1925).

2.2.7 Effect of pO_2 on hypoxanthine uptake by erythrocytes

Fresh heparinised human blood from a healthy volunteer was centrifuged for 10 minutes at 1700 g and the plasma, buffy coat and top 1/5th of red cells removed. The infranatant

cells were washed twice with 3 volumes of ice-cold saline and resuspended in 3 volumes buffer containing, in final concentration, HEPES 50 mM; NaH_2PO_4 1, 5 or 10 mM; ^{14}C-hypoxanthine 120 µM (5 Ci/mol); glucose 10 mM; and NaCl to provide a final osmolality of 290 mOsm/kg. pH was adjusted to 7.1 with NaOH. After equilibration with air at room temperature, 0.7 ml samples of the erythrocyte suspension were removed and introduced under dibutylphthalate in a 1.5 ml microfuge tube which was then closed and kept at room temperature. The pH and pO_2 of an aliquot of the erythrocyte suspension was measured. Nitrogen gas was then bubbled through the remaining suspension at room temperature and at appropriate intervals 0.7 ml aliquots were removed and treated as described above until the pO_2 had dropped to 2.7 kPa or below. All the microfuge tubes were then incubated at 37°C for 90 min with intermittent inversion to keep the erythrocytes suspended. After incubation the erythrocytes were separated from the medium by centrifugation; the dibutylphthalate settled between the heavier red cells and less dense medium. 50 µl aliquots of the pelleted erythrocytes were then lysed with 100 µl H_2O and deproteinized with 150 µl 0.6 M perchloric acid. After centrifugation and the supernatant fluid was neutralised with 2.5 M K_2CO_3 the total erythrocyte content of labelled oxypurine was determined by counting an aliquot of supernatant. The nature and quantity of individual hypoxanthine metabolites in the cells was determined using both reverse phase and anion exchange chromatography;

appropriately timed aliquots of the effluent were collected and counted.

All estimations were performed in duplicate. The difference between duplicates expressed as a percentage of the mean was found to be less than 5 percent in most cases and less than 10 percent in all cases.

2.2.8 Preparation of activated charcoal

200 g of commercial grade activated charcoal (Sigma Chemical Co., St Louis, Missouri, catalog no. C-4386) were suspended in 5 to 10 volumes of 1 M HCl and boiled for 15 to 20 minutes. After cooling and allowing the charcoal to settle the supernatant fluid was decanted and discarded. The charcoal was then washed several times with 1 M KCl until the pH of the supernatant approached 7.0 (the supernatant was decanted after allowing the charcoal to settle each time). This required many washes. The charcoal was washed 7 to 10 times with high purity H_2O and then dried in a beaker covered with cheesecloth in an oven at less than 40°C.

2.2.9 Preparation of DEAE cellulose

DEAE cellulose was prepared according to Hennessey *et al.* (1962). 40 g of commercial DEAE cellulose was suspended in 1 litre of 1 M K_2HPO_4 (pH 9.1), stirred mechanically for 1 hour and filtered with suction. This step was repeated several times until the filtrate was colourless. The adsorbent was washed further with distilled water until the

pH of the filtrate was approximately 7.0. An aqueous suspension (8 g per 100 ml) of the adsorbent was adjusted to pH 7.0 and stored at room temperature.

2.3 RESULTS

2.3.1 Effect of pH on oxypurine metabolism

Washed human erythrocytes were incubated at 37°C for 2 hours in HEPES buffered medium containing 10 mM Pi and 100 μM ^{14}C hypoxanthine at varying pH. In a parallel experiment, cells pre-incubated at the lowest pH (7.1) for 2 hours were alkalinized by the progressive addition of NaOH to give a second set of samples of varying pH which were incubated for a further period of 2 hours at 37°C. Cells from both the first and second incubations were separated from medium by centrifugation through dibutylphthalate and the distribution of labelled purine species determined by anion exchange and reverse phase chromatography.

The results of the first 2 hour incubation are given in Fig. 2.1A and show that IMP is synthesized in progressively greater amounts with decreasing pH. Minimal quantities are present after 2 hours above pH 7.5. When incubation of cells at pH 7.1 is continued at the same pH value (Fig. 2.1B) for a further 2 hours the conversion of hypoxanthine to intracellular IMP becomes essentially complete. However, subsequent alkalinization of cells pre-incubated for an initial 2 hours at pH 7.1 resulted in a reversal of this

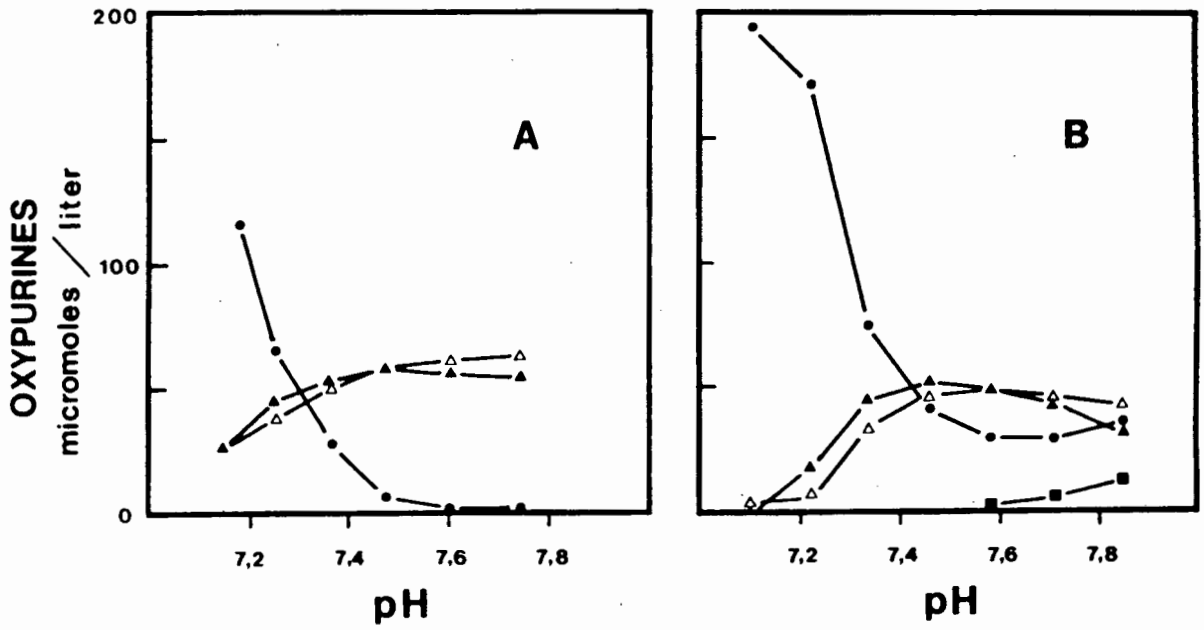


Fig. 2.1. Variation with pH in uptake and metabolism of hypoxanthine by erythrocytes.

(A), incubation for 2 h at 37°C in 10 mM Pi and 100 μM ^{14}C-hypoxanthine. (B), Pre-incubation for 2 h in 10 mM Pi and 100 μM ^{14}C-hypoxanthine, pH 7.15, followed by alkalinization and incubation for a further 2 h. ●——●, intracellular IMP; ▲——▲, intracellular hypoxanthine; ■——■, intracellular inosine; △——△, medium hypoxanthine. Units on the abscissa are in μmol/l packed erythrocytes or μmol/l medium.

process, with depletion of intracellular IMP and reappearance of hypoxanthine. At pH values above 7.6 detectable quantities of inosine accumulated. Intracellular hypoxanthine concentrations were generally similar to those in the medium, irrespective of pH, consistent with the known rapid equilibration of hypoxanthine across red cell membranes (Lassen, 1967).

2.3.2 Effect of inorganic phosphate on hypoxanthine metabolism

The effect of varying Pi concentrations on IMP formation in intact erythrocytes at a number of different pH values is shown in Figs. 2.2 and 2.3, and demonstrates the Pi dependence of IMP accumulation. No IMP accumulates at any pH in the absence of Pi in the medium. In the presence of external Pi, IMP accumulation and corresponding medium hypoxanthine depletion is dependent on pH, such that at a given Pi concentration, decreasing pH results in a progressively greater IMP accumulation.

2.3.3 Effect of pH on PRPP formation

To investigate the mechanism whereby low pH enhances IMP synthesis, erythrocytes were incubated for varying times in medium similar to that used for the experiment illustrated in Fig. 2.1A but containing no hypoxanthine. The PRPP content of the erythrocytes at varying times is shown as a function of pH in Fig. 2.4, and demonstrates that detectable

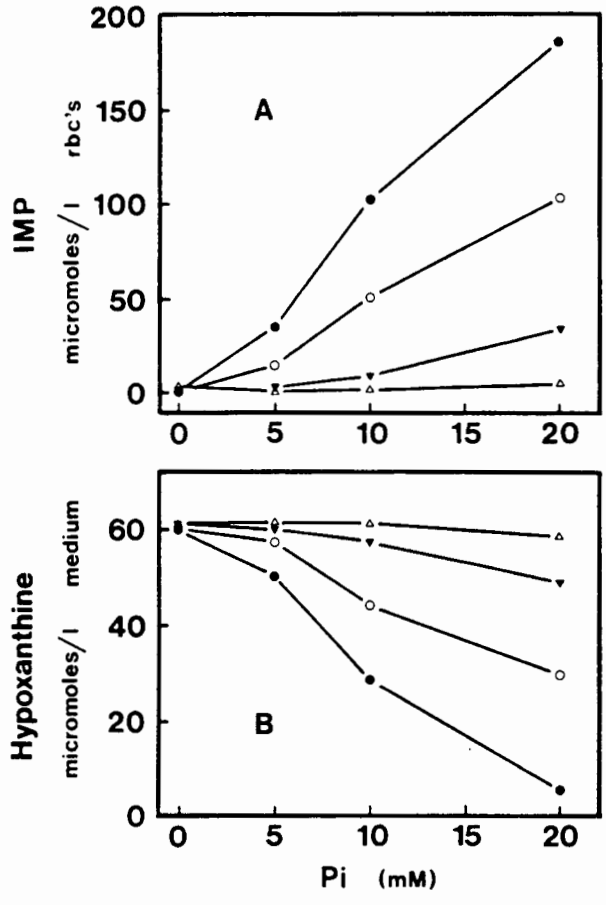


Fig. 2.2. Variation with extracellular Pi in uptake and conversion of hypoxanthine to IMP by erythrocytes.

(A), accumulation of IMP in erythrocytes and (B) depletion of hypoxanthine in medium after 1 h incubation at 37°C in 60 μM ^{14}C-hypoxanthine (1.2 Ci/mol).

●——●, pH 7.11; ○——○, pH 7.26; ▼——▼, pH 7.48;

△——△, pH 7.82. rbc's, packed erythrocytes.

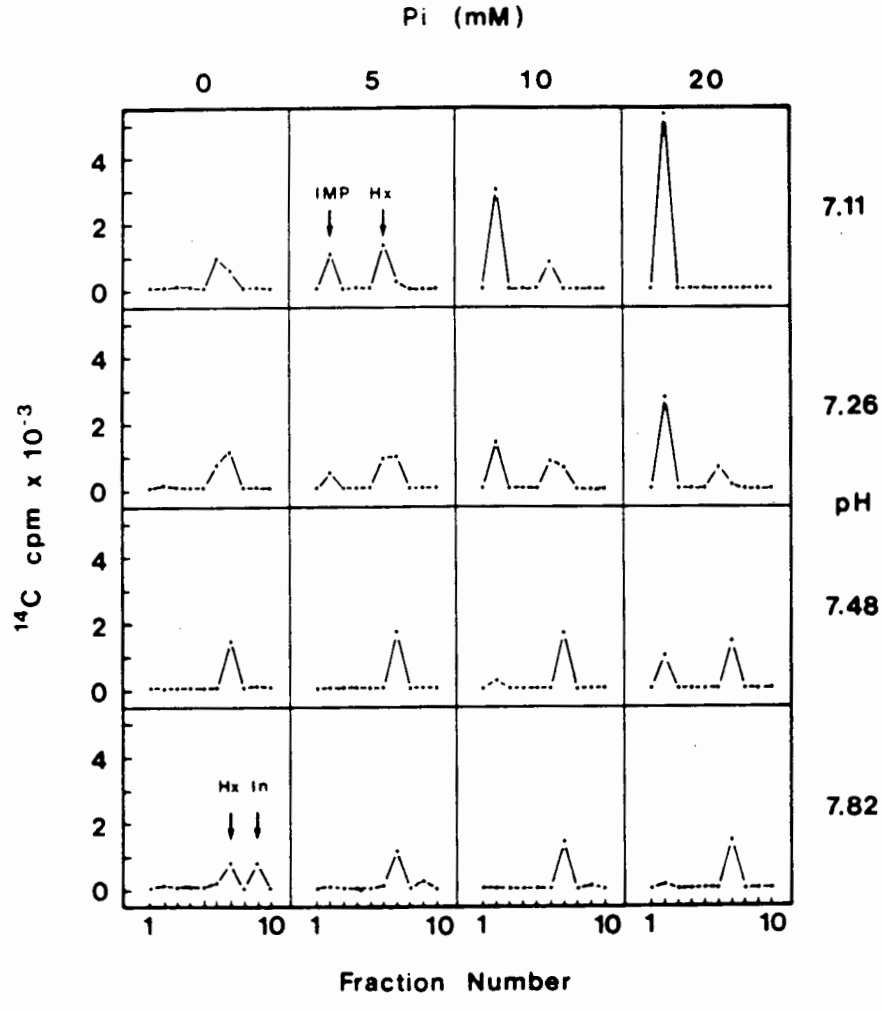


Fig. 2.3. Reverse phase HPLC of erythrocyte oxypurines. The erythrocytes were incubated with ^{14}C-hypoxanthine as described in Fig. 2.2, in media of varying pH and Pi concentration. The minimum number of fractions required for resolution of the three species were collected. Arrows show the elution positions of unlabelled markers of inosine 5'-monophosphate (IMP), inosine (In) and hypoxanthine (Hx).

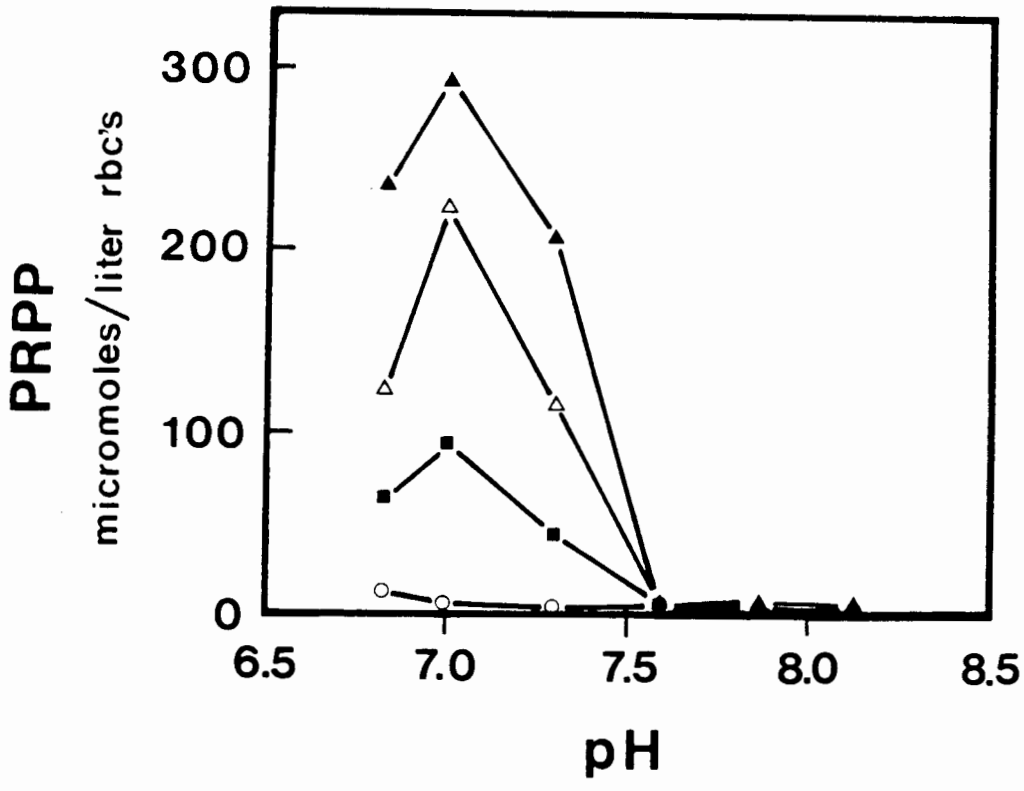


Fig. 2.4. Variation with pH in PRPP accumulation in erythrocytes.

Incubation at 37°C in 10 mM Pi. Aliquots for PRPP determination removed at hourly intervals as indicated;

○——○ (0 h); ■——■ (1 h) ; △——△ (2 h); ▲——▲ (3 h).

accumulation of PRPP occurs only at acid pH, proceeding at a maximum rate of about 100 μmoles per litre erythrocytes per hour at pH 7.0. The initial content of PRPP is less than 5 μmoles/litre, reflecting the situation in circulating erythrocytes, and remains unchanged at pH values above 7.6.

2.3.4 Effect of pH and Pi on PRPP synthetase activity

The mechanism for the pH dependence of PRPP synthesis seen above was investigated by determining the activity of PRPP synthetase in a partially purified haemolysate as a function of pH and Pi concentration. The data are given in Table 2.1 and show that whilst PRPP synthetase is highly dependent on Pi concentration for activity, it is relatively unaffected by pH, showing a broad optimum around pH 7.5. This agrees with the findings of Fox and Kelley (1971a).

2.3.5 Effect of pH on phosphate uptake and metabolism

Since the Pi concentration has such a marked effect on PRPP synthesis, both in intact cells and cell-free haemolysates (Fox and Kelley, 1971a and b), the ingress of phosphate and the intracellular Pi concentration were measured in cells incubated in media containing 10 mM ^{32}P labelled Pi at a number of different pH values. After incubation for 1 hour the cells and medium were harvested and the intracellular Pi concentration measured colorimetrically. The total uptake of phosphate was determined by measurement of ^{32}P content of both cells and medium (Fig. 2.5A). In a parallel experiment

TABLE 2.1

Effect of pH and inorganic phosphate on PRPP synthetase activity in dialysed erythrocyte haemolysate

pH	Pi (mM)	PRPP synthetase activity (μmol/hr/ml)
6.76	5	0.63 ± 0.05*
7.17	5	0.98 ± 0.14
7.73	5	1.03 ± 0.03
7.85	5	0.94 ± 0.03
8.35	5	0.82 ± 0.03
7.85	0	0.06 ± 0.01
7.85	2	0.64 ± 0.04
7.83	5	0.63 ± 0.03
7.85	10	0.73 ± 0.02
7.85	20	0.87 ± 0.06

* The values are means ± 1 SD. The determinations were carried out in quadruplicate. Pi, inorganic phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate.

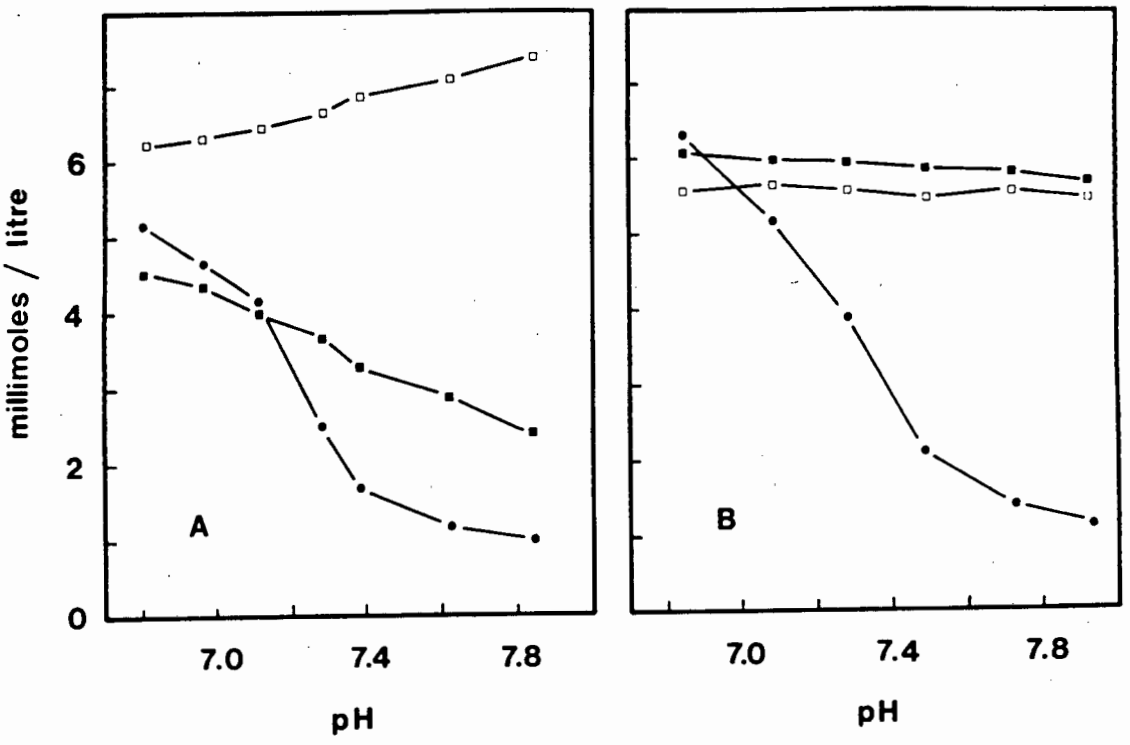


Fig. 2.5. Variation with pH in the nett influx of extracellular Pi and the free intracellular Pi concentration in erythrocytes.

(A), incubation of erythrocytes for 1 h at 37°C with 10 mM ^{32}P labelled Pi. (B) Erythrocytes pre-incubated for 1 h at 37°C in 10 mM [32P]Pi at pH 6.8 as described in (A), then divided into aliquots and the pH adjusted to values between 6.8 and 7.9. These were incubated for a further hour at 37°C. ●——●, free intracellular Pi (mmol/l packed erythrocytes); ■——■, total erythrocyte ^{32}P (amount of ^{32}Pi accumulated from the medium into both free and esterified ^{32}P phosphate) (mmol/l packed erythrocytes); □——□, medium ^{32}Pi (mM).

cells incubated for one hour at pH 6.8 under the conditions described above were subsequently divided into aliquots and the pH adjusted to provide a second set of suspensions covering a pH range of 6.8 to 7.9. These were incubated for a further hour at 37°C, then processed in the same manner as the first set (Fig. 2.5B). The results showed that acidification of cells suspended in a phosphate-containing medium results in a steep, non-linear increase in the intracellular Pi concentration. The influx of phosphate, measured by the intracellular accumulation of ^{32}P, also increass with decreasing pH but in a more linear manner and to a lesser extent, as shown by the shallower slope. This is reflected by a corresponding decrease in ^{32}P concentration in the medium. These results indicate that at higher pH values the entry of Pi into erythrocytes is slower and that much of the phosphate that does enter is no longer measurable as free Pi. At lower pH values phosphate entry is faster and is measurable as free Pi; in fact, the levels of intracellular Pi exceed those resulting from phosphate influx alone. In the second part of the experiment (Fig. 2.5B) it can be seen that alkalinization of cells preloaded with phosphate at acid pH results in a fall, progressive with increasing pH, of intracellular free Pi concentration, with little change in total ^{32}P content. This is confirmed by anion exchange HPLC of extracts from cells incubated at the extremes of pH (Fig. 2.6). A redistribution occurs in the relative amounts of ^{32}P labelled species with increasing pH; ^{32}Pi falls, with a concomitant increase in more anionic ^{32}P

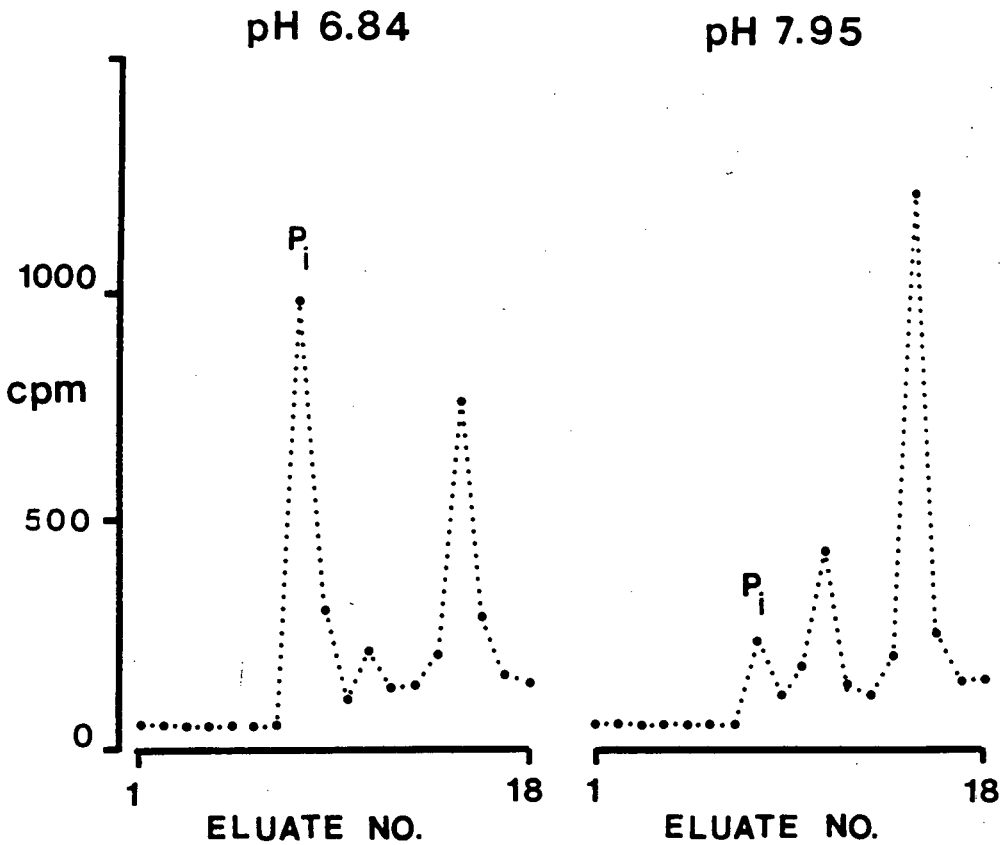


Fig. 2.6. Anion exchange HPLC of cell extracts corresponding to the most acid (pH 6.84) and alkaline (pH 7.95) incubation conditions described in Fig. 2.5B.

The distribution of ^{32}P labelled compounds is shown, with the elution position of Pi marked. The two ^{32}P containing peaks eluting after Pi were not identified, but their position suggests they represent highly anionic organic phosphates such as 2,3-DPG.

labelled compounds, the bulk of which is probably 2,3-DPG (Chiba and Sasaki, 1978). In an experiment where similarly incubated cells were transferred to a medium containing no phosphate for the second incubation step, efflux of inorganic phosphate from the cells occurred readily and was more pronounced at low pH values (data not shown).

2.3.6 Effect of pH on adenine nucleotide levels

Washed erythrocytes were incubated for 2 hours at 37°C in HEPES buffered medium containing 10 mM glucose and 10 mM Pi at varying pH. The neutralized perchloric acid extracts of the cells were chromatographed on anion exchange HPLC, and ADP and ATP levels measured relative to that of authentic standards of known concentration. The results are given in Table 2.2 and show that with increasing pH, there is an increase in the ADP, a fall in ATP and increased lactate accumulation.

2.3.7 Effect of pH on oxypurine metabolism in whole blood

To determine whether the accumulation of IMP and free Pi in erythrocytes showed the same pH dependence when suspended in plasma as in defined aqueous medium, whole blood equilibrated with CO_2 to achieve a pH of 7.0 was incubated for 90 minutes at 37°C in the presence of 10 mM Pi and 100 μM ^{14}C hypoxanthine. HPLC analysis of cell extracts prepared from aliquots removed after 5 minutes (Fig. 2.7a) and 90 minutes (Fig. 2.7b) showed progressive accumulation of IMP and

TABLE 2.2

Effect of pH on adenine nucleotide levels and lactate production

Erythrocytes were incubated at 37°C for 2 hours in 10 mm Pi at the pH values indicated. ADP and ATP levels were determined by anion exchange HPLC of cell extracts and expressed as µmol per litre of packed erythrocytes. L-lactate in the medium was measured by the enzymatic method described.

pH	RBC[ADP] (µmoles/l)	RBC[ATP] (µmoles/l)	Medium lactate mM
7.12	78	893	1.87
7.40	147	837	3.14
7.71	202	663	3.60

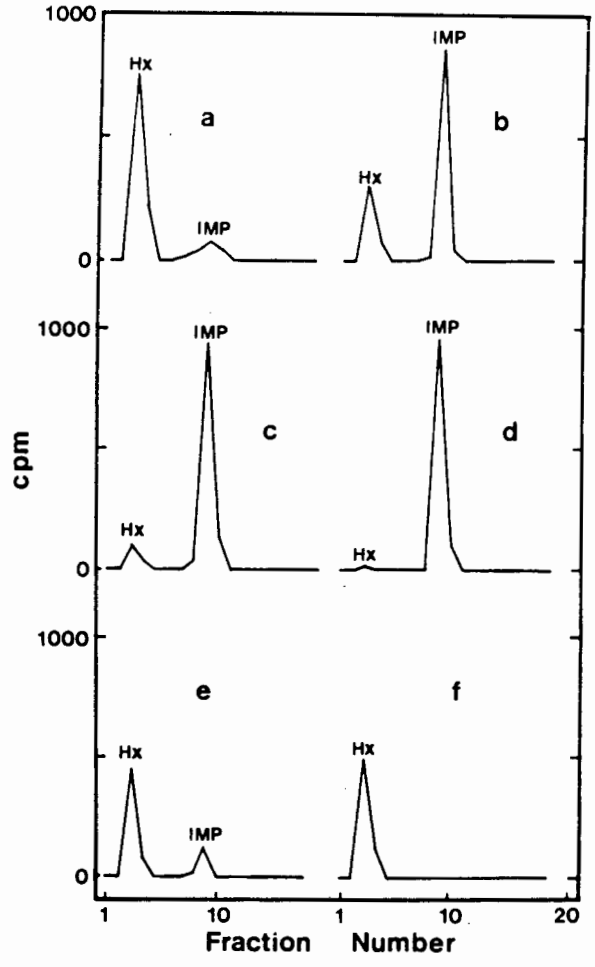


Fig. 2.7. Anion exchange HPLC of erythrocyte oxypurines. Whole blood incubated with 10 mM Pi and 100 μM ^{14}C-hypoxanthine at 37°C. pH adjusted to 7.0 by equilibration with 20% CO_2 in oxygen. HPLC analysis of intracellular purines was performed on aliquots removed after 5 minutes (2.7a) and 90 minutes (2.7b). The cells were centrifuged and resuspended in fresh autologous plasma containing no added hypoxanthine or Pi. Aliquots of cells removed before (2.7c) and after (2.7d) a further 90 minute incubation at pH 7.0. A portion of freshly resuspended cells was equilibrated with air to increase the pH to 7.8, then incubated in parallel for 90 minutes (2.7e). The plasma from this alkaline suspension was also analysed (2.7f).

decrease in hypoxanthine. Following incubation, cells were resuspended in fresh plasma equilibrated with CO_2 to maintain the pH at 7.0. Initially there was a slight decrease in cellular hypoxanthine (due to efflux into the fresh plasma) but no significant change in IMP levels (Fig. 2.7c). The resuspended erythrocytes were equilibrated with air for varying times to remove CO_2 and thus provide samples covering a pH range from 7.03 to 7.77. These suspensions were incubated for a further hour. Analysis of the plasma showed little release of radioactivity below pH 7.3 with a sharp increase above this pH (Table 2.3. HPLC analysis of plasma radioactivity from the most alkaline aliquot (i.e. the one from which the most counts were released) showed that label was confined to hypoxanthine (Fig. 2.7f). Analysis of the cells incubated at pH 7.03 and pH 7.77 revealed that at low pH remaining hypoxanthine was quantitatively converted to IMP (Fig. 2.7d), whereas at high pH most of the IMP was degraded to hypoxanthine (Fig. 2.7e) which then diffused into the plasma (Fig. 2.7f). Table 2.3 also shows that there is a decline in intracellular Pi with increasing pH. This is not accompanied by a corresponding increase in plasma Pi, indicating that the fall in red cell Pi was not due to efflux, but rather due to intracellular consumption. The rate of lactate production increased progressively with a rise in pH, reflecting a 2-fold enhancement of glycolysis over the observed pH range.

TABLE 2.3

pH effects on red cells and plasma in whole blood prelabelled with phosphate and hypoxanthine

Whole blood at pH 7.0 was incubated for 90 minutes at 37°C in the presence of 10 mM inorganic phosphate and 100 μM labelled hypoxanthine. The erythrocytes were centrifuged, resuspended in fresh plasma, equilibrated with air for various times to give the range of pH values given in the table and then incubated for 60 min at 37°C. Measurements of purine and phosphate are expressed per litre of packed erythrocytes.

pH	RBC purine (μmoles/l)	Plasma Hx (μM)	RBC Pi (mmoles/l)	Plasma Pi (mM)	Plasma lactate (mM)
7.03	131	1.6	5.48	3.86	2.19
	127	1.6	5.67	3.94	2.40
7.20	130	1.9	4.94	3.51	2.49
	127	1.8	4.99	3.64	2.56
7.29	129	2.7	4.54	3.50	2.54
	132	2.6	4.54	3.51	2.09
7.47	106	22.3	3.06	3.33	3.44
	104	23.0	3.00	3.38	3.50
7.72	77	40.9	1.92	3.21	3.82
	79	40.8	1.66	3.22	3.96
7.77	71	47.1	1.53	3.42	4.40
	72	47.6	1.70	3.50	3.91

2.3.8 Effect of pO_2 on oxypurine metabolism

To determine whether pO_2 affects hypoxanthine uptake, erythrocytes were incubated with ^{14}C hypoxanthine at pH 7.1 under conditions of varying pO_2 in the presence of three different Pi concentrations (Fig. 2.8). IMP synthesis was increased at pO_2 values less than 7 kPa at all Pi concentrations studied.

2.4 DISCUSSION

The effects of pH in the range 7.0 to 7.8 on erythrocyte oxypurine metabolism shown in this study may be related to the pH dependent changes in a number of intracellular compounds.

A fall in pH results in a rise in intracellular Pi. This may occur by two mechanisms. The first is by enhanced transport of Pi from the medium via the band 3 membrane protein which transports both divalent and monovalent anions (Passow, 1986). Transport of divalent anions, including Pi, by band 3 is increased with decreasing pH, with the maximum rate at pH 6.4 (Passow, 1969 and 1986; Gunn, 1973 and 1979; Schnell *et al.*, 1981), whereas monovalent anion transport is maximal at pH 7.0 (Funder and Wieth, 1976; Gunn, 1973). The pH dependence of phosphate transport into the red cell shown here is consistent with these characteristics of the band 3 protein. The second mechanism influencing red cell intracellular Pi concentration is the effect the prevailing

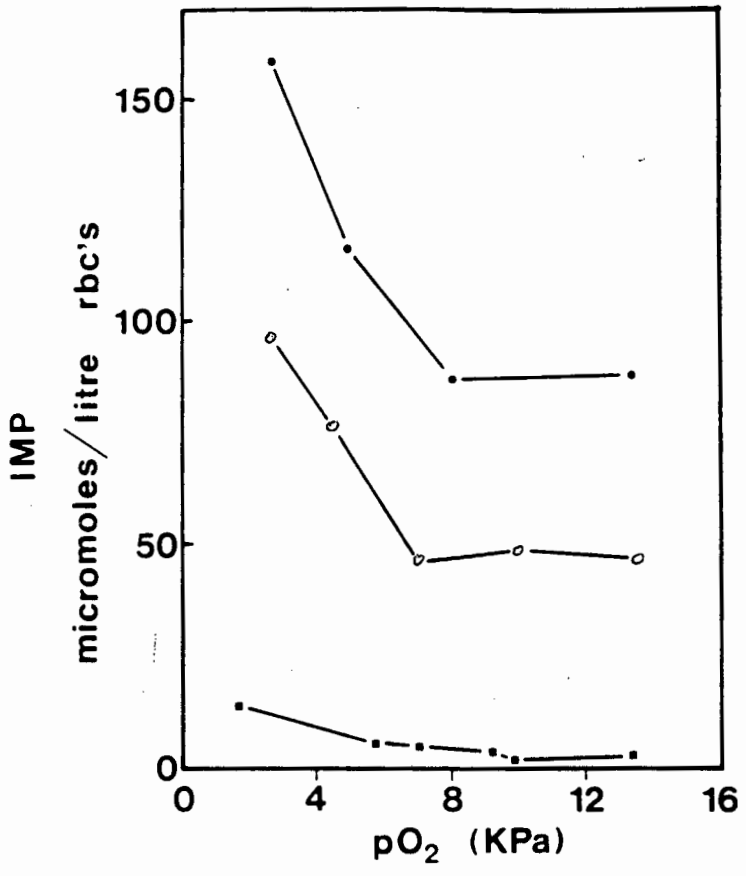


Fig. 2.8. Variation with pO_2 in the metabolism of hypoxanthine to IMP in erythrocytes.

Erythrocytes incubated for 90 minutes at 37°C with 120 μM ^{14}C-hypoxanthine at pH 7.1 in the presence of varying concentrations of Pi: (■——■ , 1 mM Pi; O——O , 5 mM Pi; ●——● , 10 mM Pi); rbc's, packed erythrocytes.

pH has on the distribution of phosphate between phosphorylated glycolytic intermediates (predominantly 2,3-DPG) and Pi. At alkaline pH values phosphate is sequestered into these organic phosphate esters (mainly 2,3-DPG): this follows the increased activities of the rate-limiting glycolytic enzymes hexokinase and phosphofructokinase under alkaline conditions (Schraufstatter and Born, 1981), and the decreased activity of 2,3-DPG phosphatase at alkaline pH (Duhm and Gerlach, 1974). A fall in pH results in a decrease in 2,3-DPG (Duhm and Gerlach, 1974; Chiba and Sasaki, 1978) and ADP, with a concomitant increase in intracellular Pi and ATP, and a fall in glycolytic flux (Minakami and Yoshikawa, 1966a and b; Newsholme and Leech, 1983), as measured by the accumulation of lactate shown in Table 2.2. These changes may be explained by the nett conversion of 2,3-DPG to lactate. Since the production of lactate from 2,3-DPG yields twice as much ATP (2 mol/mol) as production from glucose (1 mol/mol), overall glycolytic flux falls and there is an increase in the ATP/ADP ratio (Bontemps et al., 1986). Increase in pH reverses all these changes. The rise in erythrocyte ATP levels and the drop in ADP levels at acid pH values, and the reverse at alkaline pH values were confirmed and are shown in Table 2.1.

Levels of total erythrocyte 2,3-DPG fall with a decrease in pH (Chiba and Sasaki, 1978). However, much more rapid changes in the concentration of free 2,3-DPG are caused by the binding of this compound to haemoglobin (Benesch and Benesch, 1974). 2,3-DPG binds preferentially to deoxyhaemoglobin, and a 10-fold lower concentration of 2,3-DPG has been

calculated by Bunn, Ransil and Chao (1971) to exist in fully deoxygenated as compared to fully oxygenated erythrocytes. By decreasing the affinity of haemoglobin for oxygen (the Bohr effect), a fall in pH will therefore enhance the binding of 2,3-DPG to haemoglobin and also lower free 2,3-DPG levels.

It has been calculated that free intracellular magnesium ion (Mg^{2+}) concentration rises from 0.6 to 1.9 mM as fully oxygenated blood becomes deoxygenated (Bunn et al., 1971). This is a result of the competition between deoxyhaemoglobin and Mg^{2+} for the binding of organic phosphates, mainly 2,3-DPG and ATP.

The activity of three principal enzymes of purine metabolism are regulated by the compounds discussed above. These are PRPP synthetase, purine 5'-nucleotidase, and AMP deaminase. Purified PRPP synthetase shows a broad pH optimum between 7.2 and 7.4 (in contrast to PRPP synthesis in intact cells) and an absolute requirement for Pi (Fox and Kelley, 1971a and b). The results presented here confirm these findings. Mg^{2+} is required for activation of PRPP synthetase (Fox and Kelley, 1971a) although the low Km for activation (0.2 mM) implies that the variation in intracellular free Mg^{2+} concentration described above will have little regulatory influence. In contrast to Pi and Mg^{2+}, ADP and 2,3-DPG are allosteric inhibitors of PRPP synthetase activity (Hershko et al., 1969; Fox and Kelley, 1971a,b and 1972). The purine 5'-nucleotidase of red cells is a cytosolic enzyme only recently described and characterised by Bontemps, van den Berghe and Hers (1988), who showed that the activity of this enzyme is

inhibited by Pi and activated by 2,3-DPG, effects opposite to those on PRPP synthetase. AMP deaminase has been shown to be allosterically inhibited by 2,3-DPG (Askari and Rao, 1968; Lian and Harkness, 1974); in the presence of 1 mM ATP and 100 mM KCl Liam and Harkness demonstrated the Ki value to be between 4 and 11 mM. Work by Bontemps et al. (1986) has shown that the initial catabolism of human erythrocyte AMP proceeds exclusively by way of AMP deaminase under physiological conditions and upon alkalinisation, and partially by this enzyme in glucose deprivation.

The two other enzymes involved in hypoxanthine and IMP metabolism in the erythrocyte are hypoxanthine phosphoribosyl transferase (HPRT) and purine nucleoside phosphorylase (PNP). Since both IMP and PRPP accumulation show a similar dependence on pH, it can be concluded that IMP synthesis in the presence of hypoxanthine is determined by PRPP availability, and that HPRT activity is not rate limiting. The rapid conversion of IMP to hypoxanthine at alkaline pH demonstrates that PNP is not usually rate limiting, except at high pH in the absence of external phosphate. Under these conditions, intracellular Pi concentration is at a minimum, and inosine becomes detectable, since Pi is required as co-substrate for PNP (Kalckar, 1947; Kim, 1968; Lewis and Lowy, 1979). An alternative mechanism for the accumulation of inosine from hypoxanthine is by reversal of the more usual phosphorolysis (catalysed by purine nucleoside phosphorylase). This mechanism seems more likely since, when adenine was added (to deplete PRPP and thus prevent IMP formation catalysed by HPRT), inosine accumulated to the same degree

(P. Berman, unpublished observation). This Pi dependence of PNP would be consistent with the reduction in plasma inosine seen in patients with elevated plasma Pi due to renal failure (Mansell et al., 1981).

A model for the regulation of oxypurine metabolism in the red cell is illustrated in Fig. 2.9 and proposes the following: Hypoxanthine, IMP, and inosine are components of an oxypurine cycle in erythrocytes. Under conditions of low pH and high extracellular Pi, intracellular Pi concentrations rise and levels of ADP and 2,3-DPG fall, the latter effect augmented at low pO_2. The consequent stimulation of PRPP synthesis favours the conversion of hypoxanthine to IMP, and the latter accumulates due to inhibition of purine 5'-nucleotidase by Pi. Enhanced synthesis of PRPP explains the increased levels of IMP and ATP (via salvage of adenine) found in erythrocytes of patients with chronic renal failure, where blood pH is invariably decreased and plasma Pi concentration increased (Mansell et al., 1981). On returning to conditions of neutral or alkaline pH, low extracellular Pi, and high pO_2, intracellular Pi levels fall (due to efflux and inclusion into glycolytic intermediates) and 2,3-DPG and ADP levels rise. These conditions result in a concerted activation of purine 5'-nucleotidase and inhibition of PRPP synthetase, with nett conversion of IMP to hypoxanthine. Efflux of the accumulating hypoxanthine is facilitated by rapid equilibration across the red cell membrane (Lassen, 1967; Marz et al., 1979; Muller and Falkner, 1976).

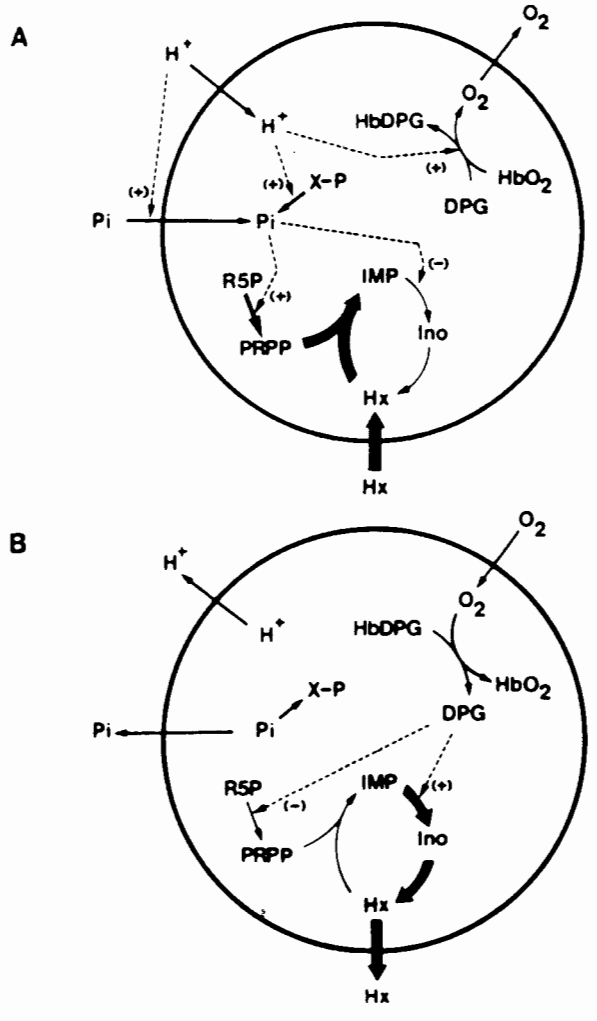


Fig. 2.9. Model depicting the factors regulating IMP and hypoxanthine metabolism in (A) conditions of acid pH, low pO_2 and high external Pi concentration, and (B) conditions of neutral or alkaline pH, high pO_2 and low external Pi concentration.

DPG, 2,3-bisphosphoglycerate; Hb, hemoglobin; Hx, hypoxanthine; Ino, inosine; IMP, inosine 5'-monophosphate; Pi, inorganic phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; R5P, ribose-5-phosphate; X-P, phosphorylated glycolytic intermediates, including 2,3-DPG.

Low pH, high Pi, and low pO_2, which favour the uptake of hypoxanthine by erythrocytes, are found in poorly oxygenated tissue (Garlick et al., 1979; Radda et al., 1983) and suggest that the erythrocyte oxypurine cycle might provide a mechanism for the transfer of hypoxanthine from less oxygenated to more oxygenated tissue in vivo. Although the Pi levels studied are somewhat in excess of physiological, a several fold increase in Pi concentration has been shown in intact ischaemic tissue by ^{31}P NMR (Radda, 1983; Radda et al., 1983; Ross et al., 1981). The cycle might also play a role in a more pathophysiological context, since in frankly ischaemic tissue hypoxanthine is known to accumulate (Osswald et al., 1977; Harkness et al., 1984; Saugstad, 1975) and has been implicated in the causation of reperfusion injury (McCord, 1985).

CHAPTER 3

TRANSPORT OF OROTATE INTO THE HUMAN ERYTHROCYTE AND ITS METABOLISM THEREIN

3.1 INTRODUCTION

Understanding of orotate physiology is generally limited to its role as an intermediate in the de novo pathway of pyrimidine biosynthesis. Most human cell cultures take up orotate poorly, if at all (Plagemann, 1971; Imura et al., 1972) and in the whole animal the liver and kidney are the predominant tissues to utilise orotate as a precursor of pyrimidine nucleotides with orotate being transported in these tissues by the organic acid transport system (Handschumacher et al., 1979). The presence of orotic aciduria in a number of pathophysiological states such as hereditary orotic aciduria (Huguley et al., 1959; Smith et al., 1961), allopurinol administration (Kelley and Beardmore, 1970), and especially in hyperammonaemia (Levin et al., 1969), demonstrate that orotate is capable of a significant flux from the liver into the plasma and ultimately, in the above conditions, into the urine.

During studies on erythrocyte pyrimidine metabolism in a patient with haemolytic anaemia caused by erythrocyte pyrimidine 5'-nucleotidase deficiency, it was found that erythrocytes showed high activity in transporting and metabolising orotate to pyrimidine nucleotides (Harley, 1978; Harley et al., 1978), and it was proposed that this was a

major pathway contributing to the erythrocyte pyrimidine nucleotide accumulation characteristic of this disorder (Harley and Berman, 1984).

The erythrocyte has long been accepted as playing a role in transport of purines between tissues in the whole organism. The presence of active pyrimidine metabolism in the erythrocyte together with the demonstrable potential for the liver to export excess orotate into the blood compartment suggests that the erythrocyte might also play a role in distributing pyrimidines synthesised in the liver to peripheral tissues.

The demonstration by Berman and Harley (1984) that the presence of erythrocytes enabled cultured fibroblasts or lymphoblasts to utilise orotate for nucleic acid synthesis lends support to this hypothesis.

Here the mechanism of orotate transport in the erythrocyte and physiological aspects affecting the metabolism of the transported orotate are investigated in the light of the findings reported in the previous chapter about the way in which pH and phosphate concentration control erythrocyte hypoxanthine and IMP metabolism. The results indicate that orotate is transported across the erythrocyte membrane by the band 3 anion transporter, and its metabolic role is influenced in a complex way by pH and phosphate concentration.

3.2 MATERIALS AND METHODS

3.2.1 Separation and extraction of erythrocytes

Fresh human venous blood from healthy volunteers was taken into lithium heparinised vacuum tubes, and centrifuged at 4°C for 12 minutes at 1700 x g. The plasma, buffy coat and top 1/5th of erythrocytes were aspirated and discarded. The remaining cells were then washed three times, with 3 volumes of ice-cold 0.9% saline.

Erythrocytes were separated from incubation medium by the technique of Wohlhueter et al. (1978). Typically, 400 µl of dibutylphthalate (Merck, Schuchardt, Germany) was added to 1 ml erythrocyte suspension in a 1.5 ml microfuge tube and centrifuged for approximately 5 seconds at 10000 x g. The pelleted erythrocytes are then separated from the supernatant medium by a discrete layer of the dibutylphthalate. A small amount of medium is carried down through the dibutylphthalate in the unstirred water layer that surrounds erythrocytes. After the supernatant had been aspirated, contaminating droplets of medium on the sides of the tubes were removed by gently layering distilled water on top of the dibutylphthalate. The distilled water and most of the dibutylphthalate were then aspirated, care being taken not to remove any of the pelleted erythrocytes. The small amount of dibutylphthalate left covering the erythrocyte pellet was deposited with the precipitate after the addition of the perchloric acid described below. The packed erythrocytes

were then resuspended in an equal volume of normal saline. The erythrocytes were then extracted with 2 volumes of ice-cold 0.6 M perchloric acid, allowed to stand for 5 min in ice and after centrifugation the clear supernatant was neutralised with 2.5 M K_2CO_3. After further centrifugation the supernatant was used for analysis.

3.2.2 Trans-membrane transport of orotate (dibutylphthalate method)

To deterine the rate of transport of orotate into human erythrocytes, each time course was commenced by adding an aliquot (usually 700 µl) of fresh, washed, packed human erythrocytes (prewarmed medium consisting of HEPES, orotate, NaCl (concentrations, and pH's adjusted with NaOH, as specified) and tracer amounts of ^{14}C-orotate (Amersham, UK.). In some experiments sucrose was substituted for NaCl. Immediately after addition of the erythrocytes to the medium the incubate was mixed thoroughly and maintained at the incubation temperature in a water bath with continuous gentle agitation. Just prior to the specified times duplicate 250 µl aliquots were removed from the incubate and transferred to microfuge tubes containing 400 µl dibutylphthalate prewarmed to the incubation temperature. At the specified times the erythrocytes were separated from the orotate containing medium by switching on the microfuge (Eppindorf, Germany) in which they had been placed. This separates the cells from the medium in less than 2 seconds (Wohlhueter et al., 1978). The pelleted erythrocytes were extracted as described above

and aliquots of the clear supernatant were counted by scintillation photometry in a Beckman LS-233 scintillation counter to determine the amount of orotate that had entered the erythrocytes. The sample of the supernatant from each of the final time points was neutralised and subjected to anion exchange HPLC.

3.2.3 Transmembrane transport of orotate ($HgCl_2$ method)

In some orotate uptake experiments a method adapted from that used by Lieu et al. (1971) was used. Incubations were carried out in HEPES-buffered saline (concentrations and pH specified). Time courses were started by adding 1.0 ml of packed, washed erythrocytes, pre-equilibrated at 37°C, to 3.0 ml of the specified ^{14}C-orotate containing buffer also pre-equilibrated at 37°C, mixing, and incubating at 37°C. At specified times 500 µl aliquots were removed and added to approximately 8 ml ice-cold $HgCl_2$-stopping solution [$HgCl_2$ (2.0 mM); Kl (1.25 mM) and NaCl (340 mM)] which prevents the transport of pyrimidine nucleotides across erythrocyte membranes (Lieu et al., 1971). Mercury, the active ingredient, combines readily with sulphydril groups in proteins, thus interfering with their function. The samples were then immediately centrifuged at 650 x g at 4°C for 60 seconds; the centrifuge took a further 90 seconds to come to standstill. The supernatant was decanted and the pelleted erythrocytes were washed with a further 8 ml of ice-cold $HgCl_2$ solution and repeating the centrifugation. Then 0.5 µl 0.9% saline was added to resuspend the cells prior to precipitation of proteins by the addition of an equal volume of 15% TCA. After centrifugation the clear supernatant was

added to 10 ml scintillation fluid (Instagel, United Technologies Packard) and counted in a Beckman LS-233 Liquid Scintillation System.

In some experiments specific inhibitors of anion transport known to act at different sites on the band 3 protein (Cabantchik et al., 1978; Falke and Chan, 1986) were employed: 4,4'-diisothiocyanostilbene-2,2'-disulfonate(DIDS) (Sigma, cat No. D-3514) (Grinstein et al., 1978; Shami et al., 1978) and alpha-cyano-4-hydroxycinnamate (CHCA) (Halestrup, 1976).

3.2.4 Metabolism of orotate by erythrocytes

Washed erythrocytes at a packed cell to medium ratio of 1:5 (v/v) were incubated in duplicate in 1.5 ml microfuge tubes at 37°C. The medium contained HEPES (50 mM), glucose (10 mM), ^{14}C orotate (10 µmolar, 61 Ci/mol) (Amersham, Buckinghamshire, England), NaH_2PO_4 (concentrations specified), with pH adjusted to specified values with NaOH, and adjusted to isotonicity with NaCl. After 4 hours incubation with constant gentle agitation the cells were separated from the medium by centrifugation through dibutylphthalate. 200 µl of the clear supernatant was removed, added to an equal volume of ice-cold 0.6 M PCA, vortexed, allowed to stand for 5 minutes and then centrifuged. The clear supernatant was neutralised with 2.5 M K_2CO_3 and analysed as specified. The pelleted

erythrocytes were extracted as described above. Pi determination was by the method of Fiske and SubbaRow (1925).

3.2.5 High Pressure Liquid Chromatography (HPLC)

Separation of the metabolites of orotate was effected by HPLC (Spectra Physics Model 3500 B) of neutralised perchloric acid extracts of erythrocytes using an anion exchange column (Hichrom APS HVP-2511) (Hichrom, Reading, England), with a linear buffer gradient developed over 10 minutes from (5 mM KH_2PO_4 (BDH, Aristar grade) pH 2.50) to (500 mM KH_2PO_4 plus 912 mM KCl, pH 3.8) at a flow rate of 1.2 ml/min. The absorbance of the effluent was monitored at 260 nm. Thirty second fractions of the effluent were collected, mixed with scintillation fluid, and counted in a Beckman LS-233 scintillation system.

3.2.6 Haemoglobin estimation

Any haemolysis that may have taken place was estimated by measuring the haemoglobin content at the beginning and at the end of an experiment using Drabkins reagent which consists of $K_3Fe(CN)_6$ (0.6 mM), KCN (0.75 mM), KH_2PO_4 (1 mM) and adjusted to pH 7.2 with KOH. To 5.0 ml reagent 20 µl of sample was added and allowed to stand for at least 5 min and the absorbance at 540 nm then read against "Drabkins" as blank. Multiplying the value thus obtained by 35.8 gives the concentration of haemoglobin in g/100 ml. In all experiments the degree of haemolysis was less than 3%.

3.2.7 Purity of orotate and purification

Specimens of ^{14}C-orotate (Amersham, U.K.) were subjected to anion exchange and reverse phase HPLC before use. If necessary, the ^{14}C-orotate was purified by HPLC, using the anion exchange column specified above. The orotate fraction was eluted using a linear gradient of NH_4COOH from 1 mM to 1.5 M over 15 minutes. After lyophilisation the orotate-containing fraction was reconstituted with H_2O, and stored at -20°C.

3.2.8 Cell-culture experiments

Normal fibroblasts were seeded in 6-well Nunc tissue plates and grown to confluence in Dulbecco's minimal essential medium (DMEM) (Gibco Ltd., Paisley, Scotland) plus 10% foetal calf serum (FCS) (Flow Laboratories, Rickmansworth, England) at 37°C and 8% CO_2 in air. Four wells were then washed with 2 ml medium containing DMEM, 10% FCS, NaH_2PO_4 (10 µM), HEPES (20 mM) and adjusted to pH 7.0 with NaOH. Four more wells were washed with the same medium except with pH adjusted to 7.4; and a further four wells at pH 7.8.

1 ml Aliquots of medium consisting of (DMEM, FCS (10%), NaH_2PO_4 (10 mM), HEPES (20 mM), Neomycin sulphate (60 mg/L), Streptomycin sulphate (100 mg/L), Sodium benzyl penicillin (100 µg/L), and ^{14}C-Orotate (0.2 µCi/ml, 61 mCi/mmol) (Amersham International, Amersham, England)) was added to

each of the 12 wells; 4 at pH 7.0, 4 at pH 7.4 and 4 at pH 7.8.

400 µl Packed washed erythrocytes were then added to 2 of each of the sets of 4 wells at each pH and incubated for 4 hours at 37°C in air.

The erythrocytes were then quantitatively removed by washing with saline and separated from the saline by centrifugation through dibutylphthalate. The erythrocytes were then precipitated with an equal volume (400 µl) of 15% TCA. The precipitated material was then dissolved in 0.1 M NaOH, scintillation fluid added, and counted in a Beckman LS-233 liquid scintillation counter.

The fibroblasts (remaining in the wells) were then washed three times with 5% TCA to precipitate the protein. The precipitate was then dissolved in 1 ml NaOH (0.1 M), neutralised with 0.5 ml 6.6% acetic acid, dissolved in scintillation fluid and counted.

3.3 RESULTS

3.3.1 Transport

In order to determine the basic kinetics of orotate transport across the erythrocyte membrane, washed erythrocytes were incubated for various times at different concentrations of ^{14}C-labelled orotate. To prevent

metabolism of the transported orotate, phosphate and glucose were omitted from the incubation medium, thus avoiding accumulation of the PRPP required for metabolism of orotate to OMP. HPLC analysis of the labelled erythrocytes confirmed (data not shown) that under these conditions no conversion of orotate to products occurred. The results (Fig. 3.1a) show that uptake is linear up to at least 5 min and the secondary plot (Fig. 3.1b) of the slopes representing initial velocity of uptake, Vi, against orotate concentraiton, shows that uptake is non-saturable up to a concentration of at least 4.6 mmolar, a value close to the maximum solubility of sodium orotate in water.

A competition experiment was performed to determine if orotate is transported by the nucleoside transporter (Jarvis, 1986). Erythrocytes were labelled with orotate for varying times in the presence and absence of 10 mmolar uridine. The accumulation of orotate with time (Fig. 3.2) was unaffected by the uridine indicating that orotate does not cross the erythrocyte membrane via the nucleoside transporter. Since orotate is an organic anion, competition and inhibitor experiments were performed to find out whether orotate is transported by the 95 K band 3 protein, the general anion transporter of the erythrocyte membrane (see section 1.6). Both HEPES and bicarbonate are frequently used anionic buffers and both inhibited orotate transport at concentrations typically employed (Table 3.1). The effect of two specific inhibitors of anion transport in the

Fig. 3.1 Kinetics of orotate uptake by erythrocytes.

700 µl packed erythrocytes were added to 3 ml medium at 37°C consisting of 50 mM HEPES (adjusted to pH 7.4 with NaOH), ^{14}C orotate at the appropriate concentration and adjusted to isotonicity with NaCl.

a) Plot of orotate accumulation with time at external orotate concentrations ◇——◇ , 0.23; ◆——◆ , 0.46; △——△ , 0.69; ▲——▲ , 0.93; □——□ , 1.85; ■—— , 2.78; ○——○ , 3.70; and ●——● ,4.63 millimolar. Each measurement was performed in quintuplicate with the points representing the mean of these measurements. The lines were fitted by the least squares method giving coefficients of variation in excess of 0.99 in all cases.

b) Plot of the slopes from (a) versus the corresponding orotate concentration.

Fig. 3.1a

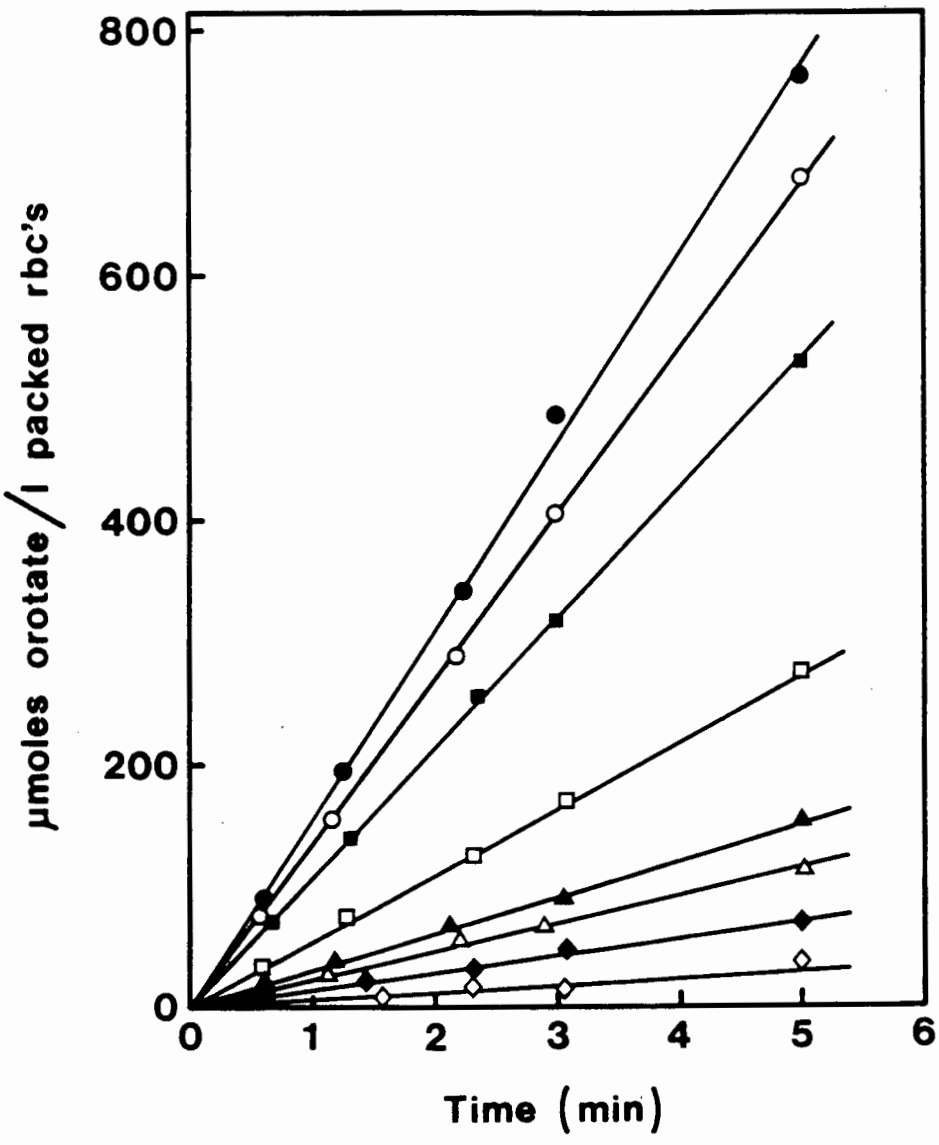
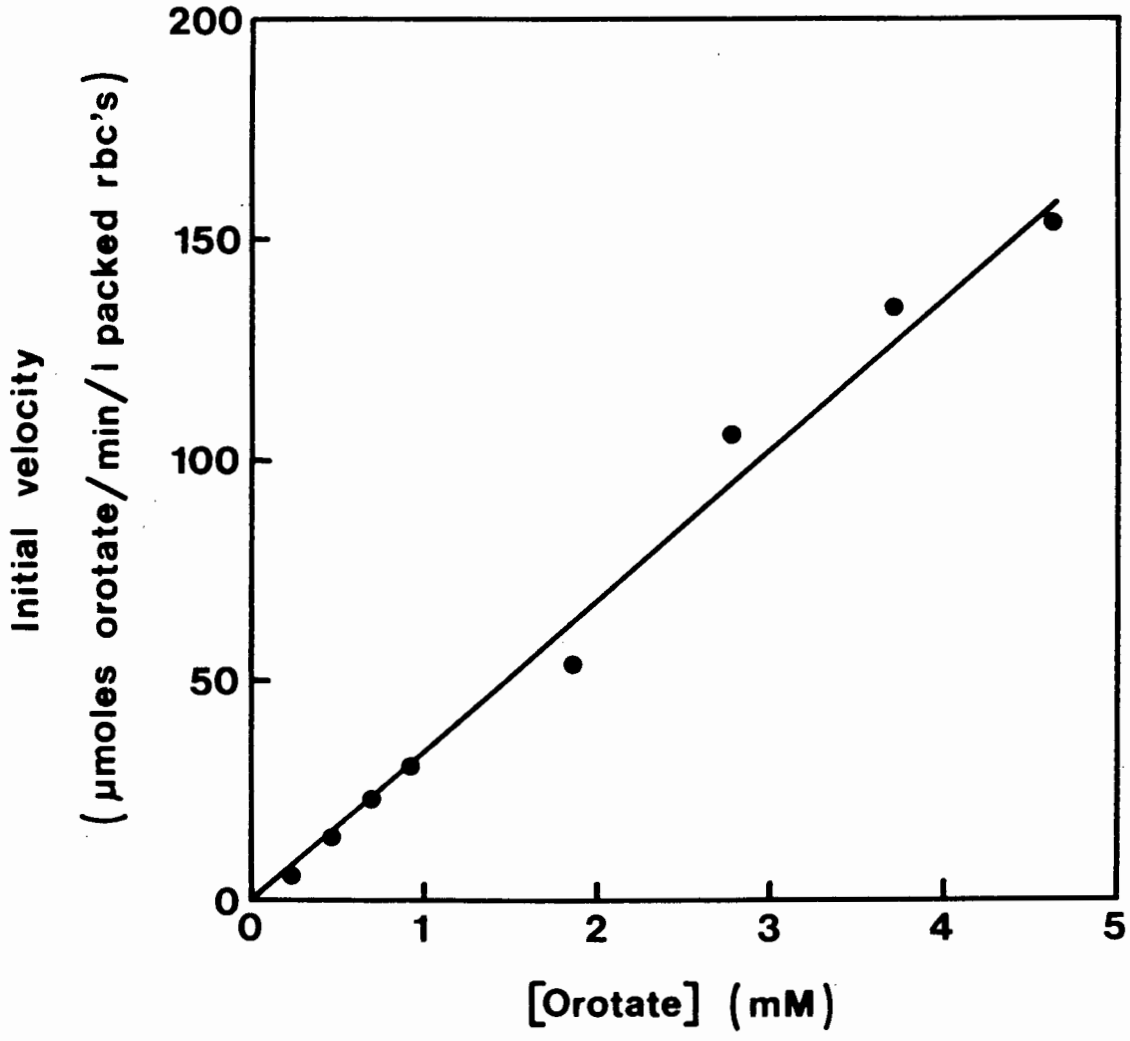

800
600
400
200
0
µmoles orotate/l packed rbc's
0
1
2
3
4
5
6
Time (min)

Fig 3.1b


Initial velocity
(μmoles orotate/min/l packed rbc's)
200
150
100
50
0
0
1
2
3
4
5
[Orotate] (mM)

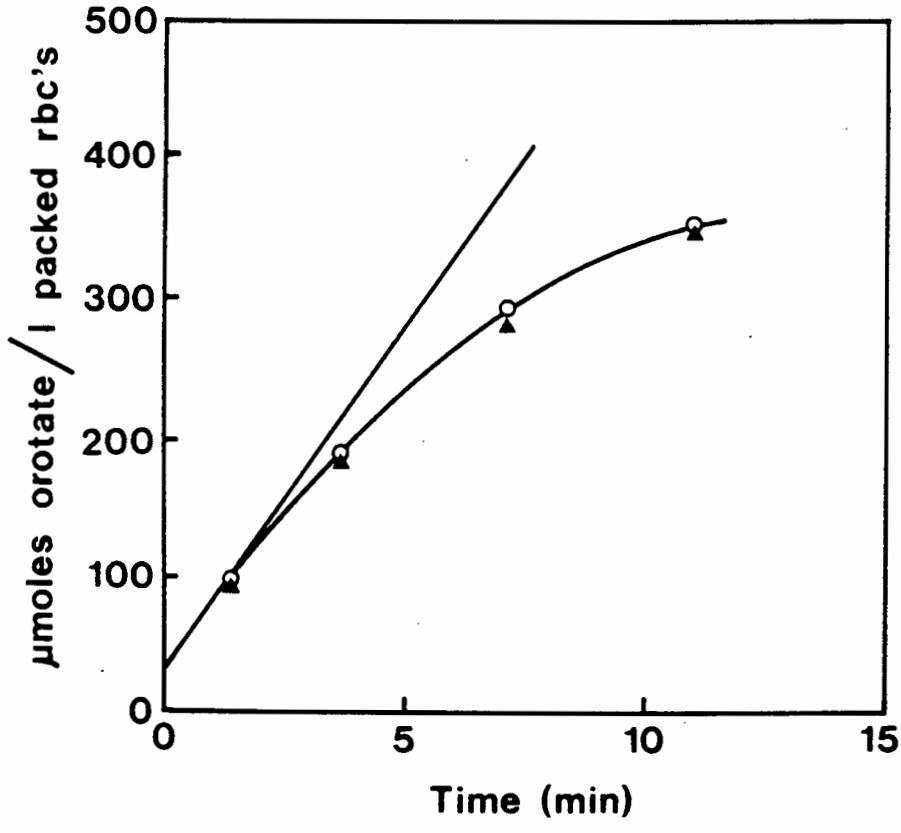


Fig. 3.2. Effect of uridine on uptake of orotate by erythrocytes. 1 ml packed erythrocytes were added to 3 ml medium containing 1.74 mM ^{14}C orotate, 20 mM HEPES, pH 7.4 adjusted to isotonicity with NaCl, in the presence or absence of uridine. Aliquots were taken and delivered into $HgCl_2$ stopping solution at the indicated times. Points represent the mean of duplicate values which varied from the mean by less than 5% in all cases. ▲——▲ , in absence of uridine; O——O , in presence of 10 mM uridine.

Table 3.1

Effect of anions on orotate transport

250 µl packed erythrocyte were added in duplicate to 3 ml saline-free medium at 25°C containing 1 millimolar ^{14}C-orotate, and the appropriate quantity of competing anion at pH 7.4, and adjusted to isotonicity with sucrose. Aliquots were taken at 1 minute intervals for measurement of orotate uptake by the dibutylphthalate method and Vi was obtained from the slopes of the resulting plots as in Fig. 3.1. The duplicated points all fitted straight line plots with correlation coefficients better than 0.97.

ADDITIONS	OROTATE Vi (µmoles/min/l packed cells)	% OF CONTROL
No additions	370	100
HEPES 5 mmolar	143	39
" 20 mmolar	117	32
" 50 mmolar	93	25
Bicarbonate 24 mmolar	70	19

erythrocyte, DIDS and CHCA are given in Table 3.2 and demonstrate that transport of orotate is inhibited by both compounds.

In both competitor and inhibitor experiments, transport of orotate was so rapid in the presence of low concentrations of competing anions that it was necessary to perform the experiments at 25°C instead of 37°C in order to obtain straight line plots for determination of Vi. Hence in a medium containing 20 mmol HEPES but lacking chloride anions, transport of orotate at 37°C was too rapid to obtain linear plots of orotate uptake with time, although the initial rate of uptake at 1 mmolar orotate could be assigned a minimum value of 530 μmoles/min/l packed erythrocytes, a value 15 times greater than the corresponding rate in the presence of 140 mmolar chloride. Fig. 3.3 illustrates the results of orotate uptake in the absence of chloride at 25°C, where the Vi for a given concentration of orotate is still 3.2 times greater than that in 140 mmolar chloride at 37°C.

The effect of pH on the initial velocity of orotate uptake was measured across the range of 6.2 to 7.8 (Table 3.3). There was a broad pH optimum centered at about pH 6.5 but transport was still 80% of maximum at pH 7.4.

The single, selective, anion transport site on band 3 is alternating in nature and proceeds via a ping-pong mechanism (Gunn and Frohlich, 1979; Falke and Chan, 1985). Under

Table 3.2

Effect of anion transport inhibitors on orotate transport

200 µl packed erythrocytes were added in duplicate to 3 ml medium at 25°C, containing the appropriate concentrations of inhibitor and 1 mM ^{14}C-orotate, 20 mM HEPES (pH 7.4), adjusted to isotonicity with sucrose. Vi was determined as described in Table 3.1. Since DIDS is light-sensitive the manipulations were performed in very dim light.

ADDITIONS	OROTATE Vi (µmoles/min/l packed cells)	% OF CONTROL
Exp. I		
No additions	106	100
CHCA, 100 µmolar	49	47
CHCA, 500 µmolar	39	37
Exp. II		
No additions	104	100
DIDS 1 µmolar	54	52
DIDS 5 µmolar	7	7

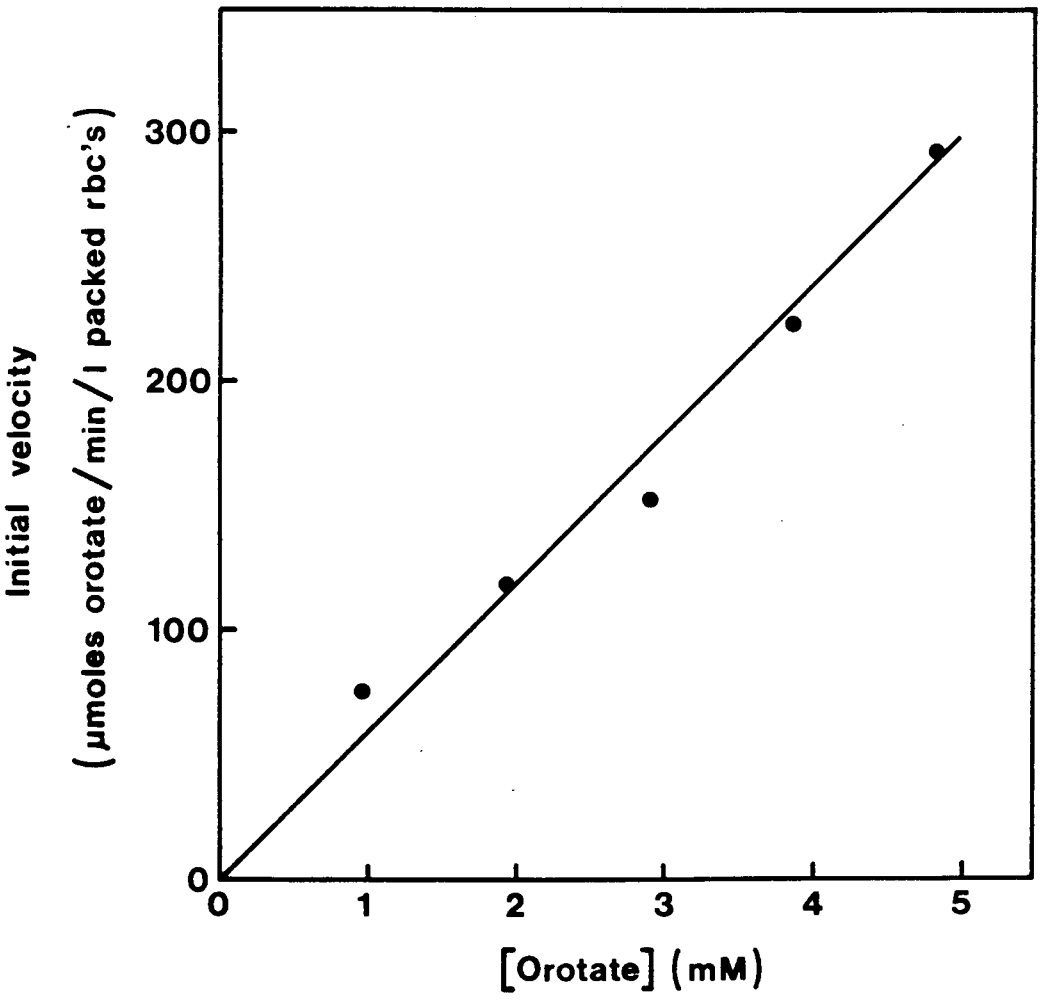


Fig. 3.3. Effect of chloride-free medium on rate of uptake of orotate by erythrocytes. 500 μl packed erythrocytes were added to 4 ml medium at 25°C containing varying concentrations of ^{14}C-orotate, 20 mM HEPES, pH 7.5 and adjusted to isotonicity with sucrose. Aliquots were taken at 1 min time intervals for determination of orotate uptake and for production of primary plots as in Fig. 3.1a. These were all straight line plots with correlation coefficients better than 0.98 and their slopes were used for production of the secondary plot of initial velocity versus orotate concentraiton shown here.

Table 3.3

Effect of pH on orotate uptake by erythrocytes

250 µl packed erythrocytes were added in duplicate to medium at 37°C containing 1 mmolar ^{14}C-orotate, 20 mmolar HEPES, brought to the specified pH with NaOH and adjusted to isotonicity with NaCl. Vi values were obtained from the linear time course plot as described above.

pH	OROTATE Vi (µmoles/min/l packed cells)
6.26	38
6.66	40
7.04	36
7.40	32
7.76	31

conditions where there is a relative paucity of anions in the incubation medium, there will be a positive inside membrane potential (Nicholls, 1982). The band 3 transport mechanism should therefore result in the intracellular accumulation of a compatible anion to occur at concentrations above those in the medium. Erythrocyte Cl- concentration is 50 mM (Caraway, 1962) and Fig. 3.4 demonstrates a concentrative uptake for orotate in the absence of chloride in the medium. After 10 minutes, values for intracellular orotate concentrations reached levels six times greater than the initial external orotate concentration. The orotate concentration in the erythrocyte took about 3 hrs to reach equilibrium values, presumably reflecting the gradual dissipation of the positive inside membrane potential. When erythrocytes were pre-incubated in chloride-free medium containing 1 mM orotate for 16 hours (Fig. 3.5) this allowed internal and external orotate concentrations to equilibrate, and on addition of a trace quantity of ^{14}C-orotate, equilibration occurred rapidly with a half-time of about 7 minutes. No concentrative uptake was observed.

3.3.2 Metabolism

Since metabolism of the orotate transported into the erythrocyte is dependent on PRPP, the following experiments were performed in glucose and phosphate containing media. The dependence of orotate uptake and metabolism in pH and inorganic phosphate (Pi) concentration was studied using HPLC analysis of PCA extracts of cells incubated in medium

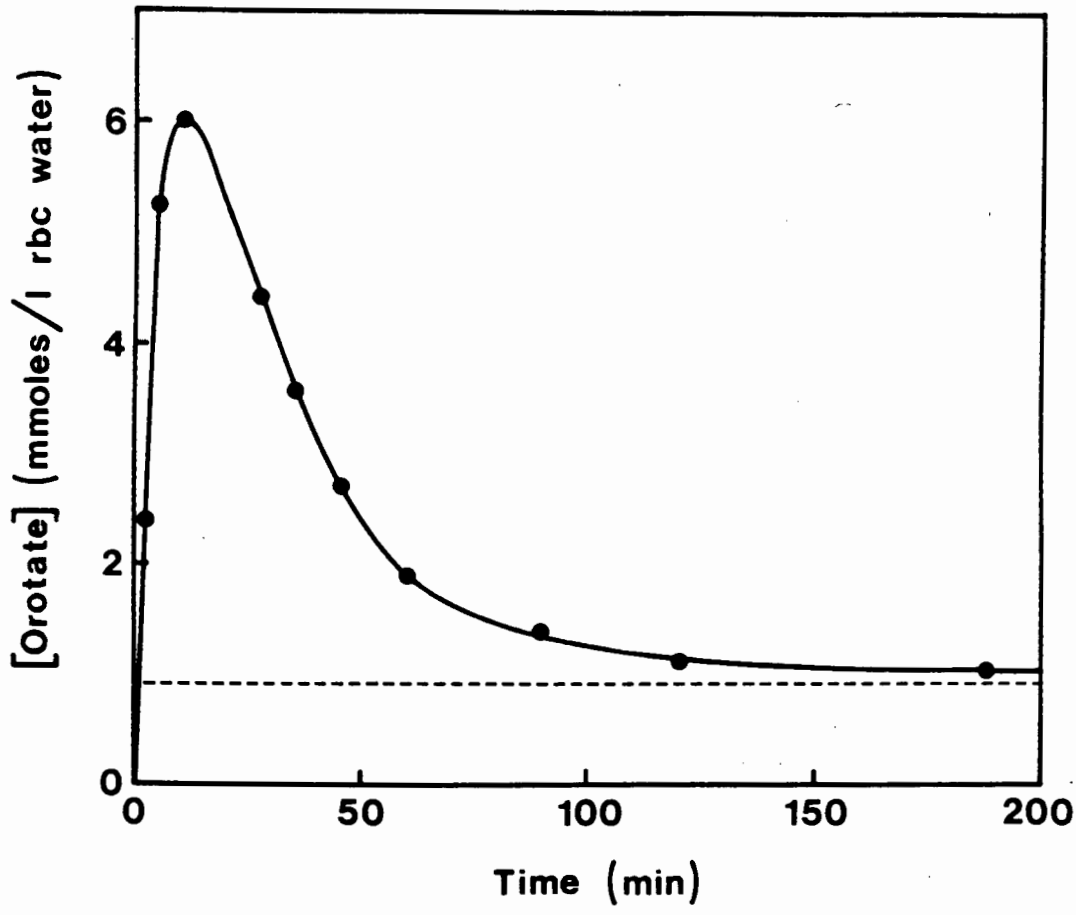


Fig. 3.4. Intracellular accumulation of orotate in chloride-free medium. 500 µl packed erythrocytes were added to 5 ml of medium at 37°C containing 1 mM ^{14}C-orotate, 10 mM HEPES, pH 7.4, and adjusted to isotonicity with sucrose. 200 µl aliquots were removed at the specified intervals for determination of packed erythrocyte orotate concentrations. To express the results in mmol/l erythrocyte water, a ^{14}C-sucrose dilution was used to determine that under the experimental conditions used, packed erythrocytes consisted of 70% erythrocytes and 30% extracellular fluid, furthermore, under isotonic conditions erythrocytes contain 65-70% water by volume (Brewer, 1978).

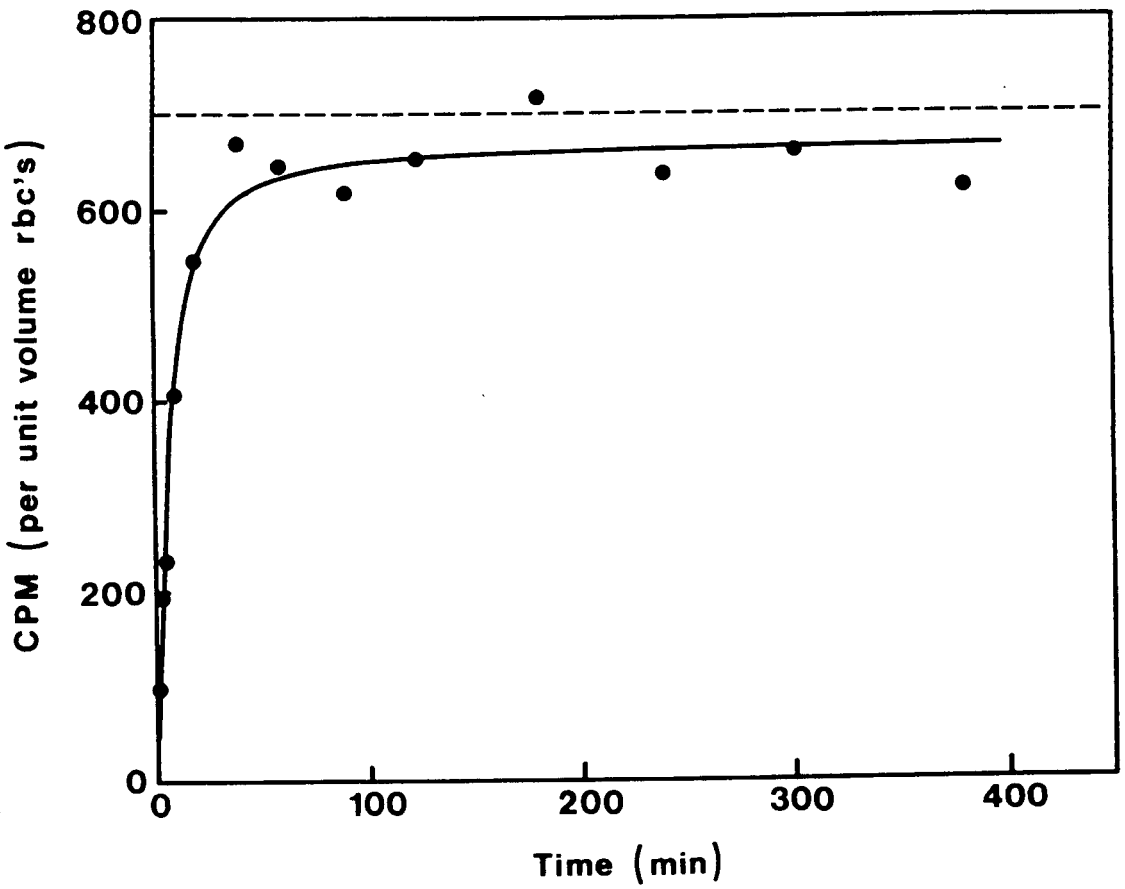


Fig. 3.5. Intracellular accumulation of orotate after 16 hours pre-incubation in chloride-free medium containing 1 mM orotate, 20 mM HEPES, pH 7.4, and adjusted to isotonicity with sucrose. A tracer amount (5×10^{-2} μCi/ml, equivalent to 4 μmolar) of ^{14}C-orotate was added and duplicate samples taken for determination of radioactivity at the specified time intervals.

containing labelled orotate at a concentration of 10 μmolar. Fig. 3.6 compares the accumulation of orotate in various product species as a function of pH and at varying Pi concentrations. Accumulation of label in nucleotides is poor in the absence of external phosphate, with UDP sugars being the predominant nucleotide species accumulating. However, significant quantities of uridine are formed under these conditions, and these quantities are not greatly affected by pH. Low pH favours the accumulation of label in nucleotides. Increasing phosphate concentrations increase the accumulation of label in UTP at all pH values. Accumulation in UDP sugars is somewhat different with a lesser quantity of label accumulating at high phosphate concentrations than at intermediate levels. At alkaline pH (7.8) uridine production increases with increasing phosphate concentration, whereas at acid pH values this relationship breaks down, with the smallest quantities of uridine being formed at the highest external Pi concentrations. The total orotate uptake is therefore a complex function of pH and Pi concentrations.

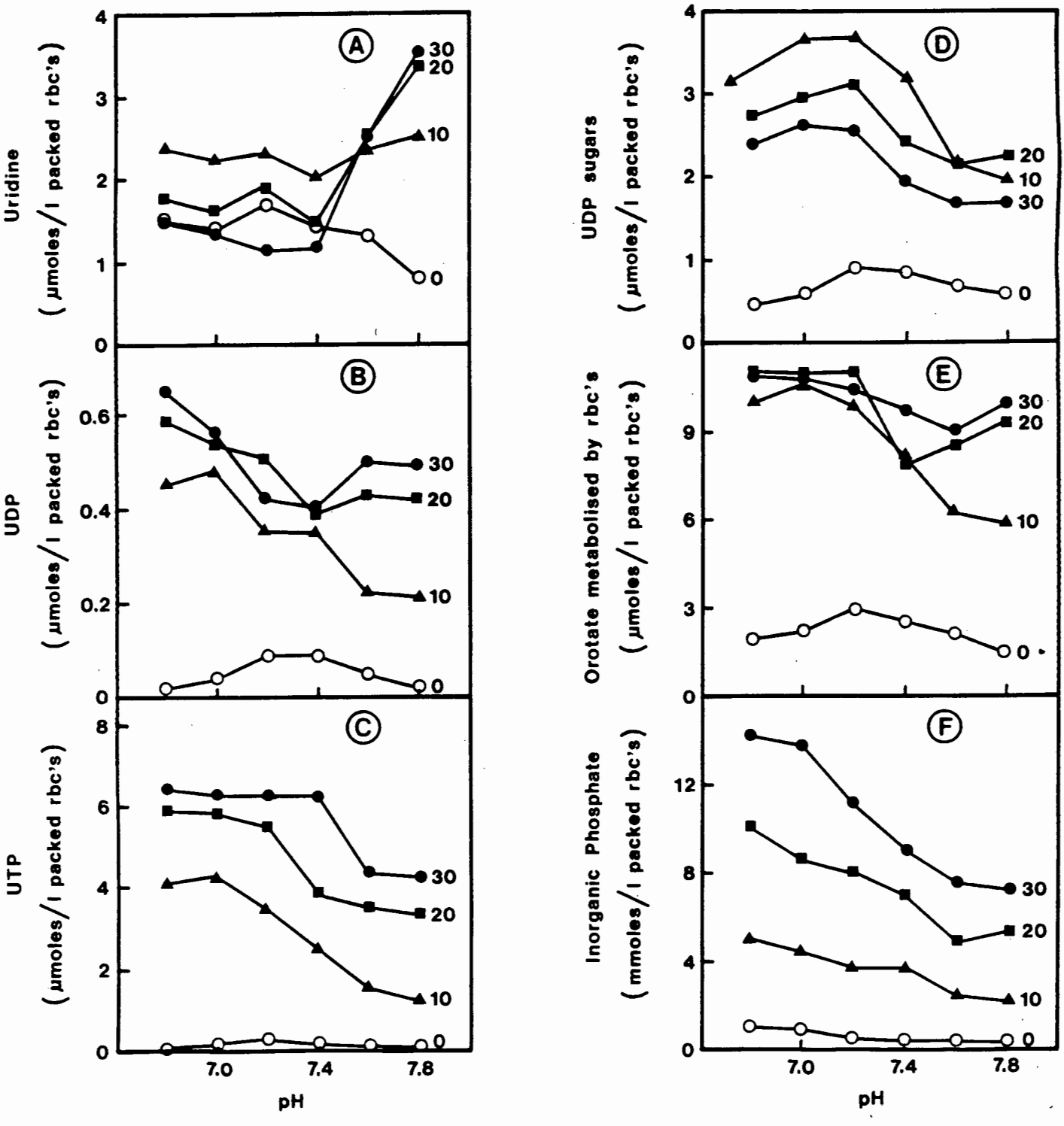
Since the activity of both pyrimidine 5'-nucleotidase and PRPP synthetase are regulated by phosphate, the former being inhibited (Torrence and Whittaker, 1981) and the latter activated (Fox and Kelley, 1971a and b), levels of intracellular inorganic phosphate were also measured in the above experiment at the varying conditions of pH and external Pi. The results in Fig. 3.6 show that intracellular

Fig. 3.6. Effect of pH and Pi on orotate metabolism in erythrocytes. 200 µl fresh, washed, packed erythrocytes were incubated for 4 hours at 37°C in 850 µl medium containing 10 mM glucose, 10 µm ^{14}C-orotate, 20 mm HEPES, NaH_2PO_4 at the specified concentrations. pH was adjusted with NaOH, and the solution was then adjusted to isotonicity with NaCl. At the end of the incubation period, cells were separated from medium by the dibutylphthalate method, and phosphate and the metabolites of orotate were quantitated in neutralised PCA extracts.

○——○, 0 mM Pi; ▲——▲, 10 mM Pi; ■——■, 20 mM Pi;

●——●, 30 mM Pi.

Fig. 3.6


A
B
C
D
E
F
Uridine (μmoles/l packed rbc's)
UDP (μmoles/l packed rbc's)
UTP (μmoles/l packed rbc's)
UDP sugars (μmoles/l packed rbc's)
Orotate metabolised by rbc's (μmoles/l packed rbc's)
Inorganic Phosphate (mmoles/l packed rbc's)
30
20
10
0
7.0
7.4
7.8
pH

phosphate concentrations increase proportionately with decreasing pH.

Orotate is known to enter most nucleated cell species only relatively slowly (Berman and Harley, 1984). To confirm Berman and Harley's findings that erythrocytic conversion of orotate to uridine made it available to nucleated cells, cultured human skin fibroblasts were labelled with ^{14}C-orotate in the presence and absence of erythrocytes at varying pH. The results are given in Table 3.4 and show that the presence of erythrocytes increased the labelling of fibroblast nucleic acids by greater than 10 fold at all pH values.

3.4 DISCUSSION

Here it is shown that orotate enters erythrocytes with non-saturable kinetics and with a capacity of 190 μmoles/l packed cells/min at a concentration of 4.6 mmolar. This indicates that orotate crosses the erythrocyte membrane by either simple or unsaturated facilitated diffusion. Saturation kinetics could not be determined because of the low solubility of orotate (10.8 mM per litre water at 25 °C). The presence of competition for transport by a number of anions and the lack of competition by uridine is indicative of transport by a general anion transporter (facilitated diffusion), with the ability for concentrative uptake in the absence of other external anions being compatible with transport via a ping-pong mechanism (Falke and Chan, 1985). Inhibition of transport by the specific band 3 inhibitors DIDS and CHCA confirm that transport is via the band 3 anion transporter. This explains the lack of significant uptake of orotate by most differentiated tissues (Plagemann, 1971; Imura et al., 1972; Berman and Harley,

Table 3.4

Effect of erythrocytes on incorporation of labelled orotate into fibroblasts

Cultured human skin fibroblasts were labelled with ^{14}C-orotate in the presence (test) and absence (control) of erythrocytes and at varying pH. See Methods (3.2.8) for details.

pH	Fibroblast acid precipitable material (cpm)	
	Control	Test
7.0	91	890
7.4	72	769
7.8	50	603

1984) which lack the intact band 3 protein (Kay et al., 1983; Drenckhahn and Zinke, 1984). However, the demonstration of band 3 in rat hepatocytes (Cheng and Levy, 1980) provides a mechanism for the orotate transport which has been observed in liver (Handschumacher and Coleridge, 1979).

Fig. 3.7 is a simplified diagram of the metabolism of orotate in the human erythrocyte and provides a focus for the following discussion. Changes in pH and inorganic phosphate concentrations have marked effects on the relative quantities of metabolic products produced by the erythrocyte from orotate. Since both decreasing pH, and increasing external phosphate concentration result in increased intracellular phosphate concentrations, an effect seen in Fig. 3.7 and also reported previously (Berman et al., 1988), the increase in orotate metabolised with increasing phosphate concentration, an effect augmented by lowering the pH, is most easily explained by the allosteric activation of PRPP synthetase by phosphate (Fox and Kelley, 1971a and b). The increase in levels of UTP with decreasing pH may be the consequence both of increased PRPP availability for the formation of uridine nucleotide from orotate, and decreased conversion of UMP to uridine by pyrimidine 5'-nucleotidase, which is known to be inhibited by phosphate (Torrence and Whittaker, 1981; Angle et al., 1985). The accumulation of UDP sugars is optimal at a phosphate concentration of 10 mmolar, which is unexplained but would be compatible with an inhibitory effect of phosphate on CTP synthetase.

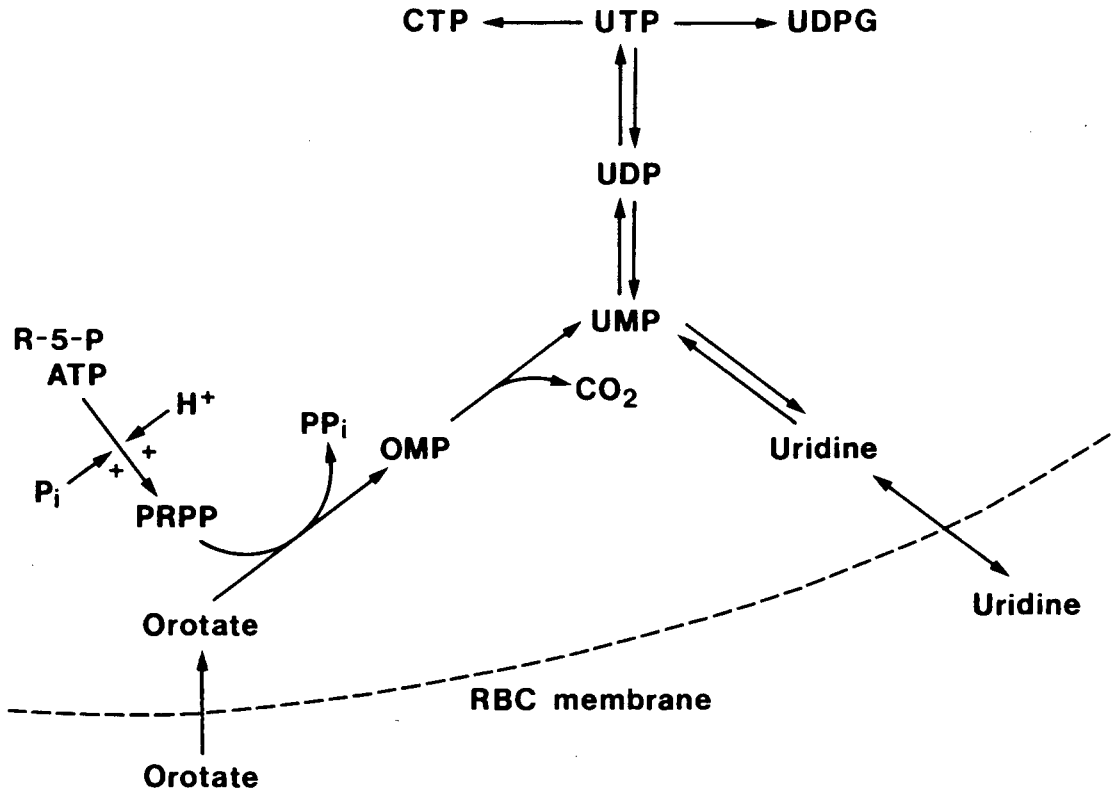


Fig. 3.7. Simplified diagram of the metabolism of orotate in the human erythrocyte.

See index for abbreviations.

In the studies on the regulation of erythrocyte hypoxanthine metabolism by pH and inorganic phosphate described in Chapter 2, no accumulation of PRPP in erythrocytes was observed when incubated at pH values of 7.6 and above in the presence of 10 mmolar phosphate and no added hypoxanthine, although accumulation was readily demonstrable at pH values below 7.4. If the lack of PRPP accumulation under these conditions is interpreted as a cessation of PRPP synthesis then this result is not compatible with the readily demonstrable metabolism of orotate, which is a PRPP utilising reaction, in the current experiment at alkaline pH values. This apparent paradox can be resolved if one assumes that even in the absence of added hypoxanthine and demonstrable intracellular IMP there are sufficient quantities of hypoxanthine and/or IMP to maintain a PRPP wasting cycle at alkaline pH values. The cycle is interrupted at acidic pH values as phosphate levels rise and inhibit 5'-nucleotidase, an effect augmented by the decreasing levels of 2,3-bisphosphoglycerate which accompany decreasing pH (Bontemps et al., 1986).

Earlier studies on orotate uptake in erythrocytes were characterised by large variations in the relative quantities of the metabolic products between different experiments (Harley et al., 1986). Now that there is a better understanding of the very fine regulation of the erythrocyte oxypurine cycle by pH and phosphate (Chapter 2; Berman et al., 1988), with PRPP dynamics being markedly altered by pH

changes as small as 0.1 units on either side of neutrality, it is easier to obtain reproducibility, by maintaining erythrocytes in mildly acidic conditions (pH 7.2) and defined phosphate concentrations, and using constant pre-incubation times, since PRPP accumulates progressively under such conditions.

The kinetics of orotate uptake by erythrocytes and its eventual release as uridine provides a role for the erythrocyte in the transport and distribution of pyrimidines to peripheral tissues, and this model is shown in Fig. 3.8. The model shows the capability of the liver to export orotate produced by the de novo pathway. This step has been demonstrated experimentally using differentiated hepatoma cells (Harley et al., 1986) and can be inferred in vivo by the demonstrable orotic acid anion of hyperammonaemia. It is a consequence of the stimulation by ammonia of de novo synthesis by mitochondrial carbamoyl phosphate synthetase I, which is predominantly a liver-specific enzyme. In the next step, the erythrocytes take up the orotate secreted by the liver into the circulation, convert it to an intermediate buffer store of uridine nucleotides, whose distribution is a function of pH and phosphate concentration, and eventually release it as uridine, which is a readily available form of pyrimidine for utilisation by peripheral nucleated cells. The enhancement of uptake of labelled orotate into nucleic acids of cultured cells by erythrocytes has been demonstrated experimentally by Harley et al. (1986), and confirmed here.

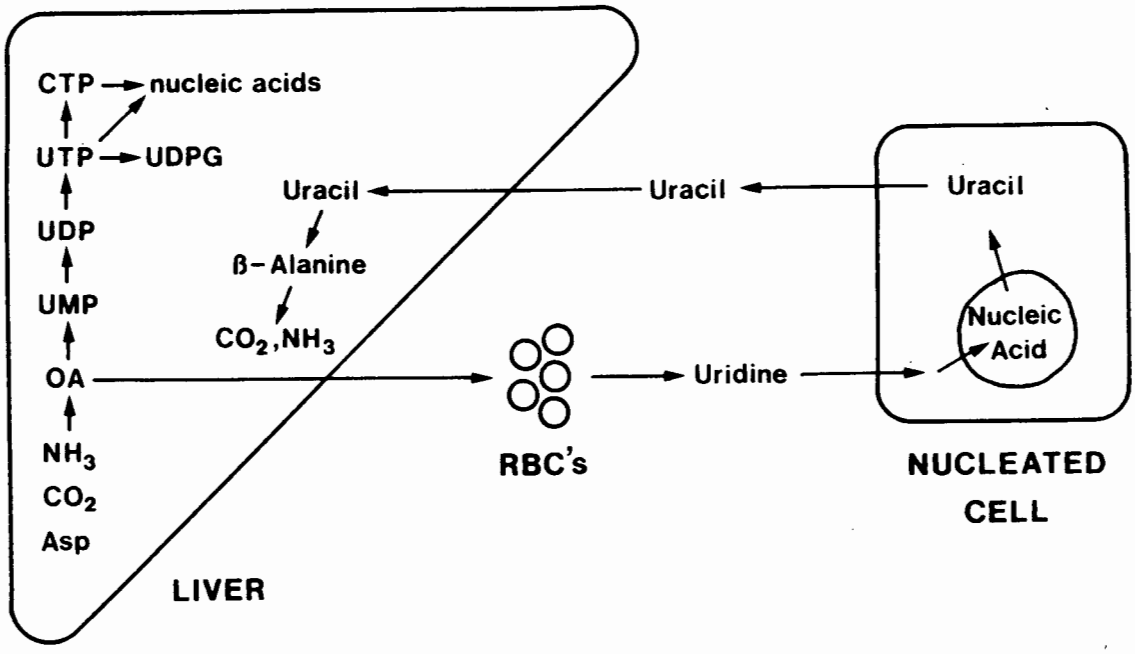


Fig. 3.8. Model of whole body pyrimidine circulation.

Asp, aspartate; OA, orotate; RBC's, erythrocytes.

The degradative half of the cycle depicted in Fig. 3.8 proposes that uracil is the predominant degradative form of pyrimidines produced by peripheral cells, and their ultimate metabolic fate is complete catabolism in the liver to CO_2 and water (Pihl and Fritzson, 1955). Whereas this is an attractive pathway logically, since there is little, if any, re-utilisation of uracil by mammalian cells, it is possible that a major degradative metabolite is uridine (and cytidine) since the predominant fate of uridine when taken up by the liver is also degradation via uracil (Harley and Losman, 1981).

The metabolic abnormalities in a number of disorders of pyrimidine metabolism are consistent with this model. The orotic aciduria of hyperammonaemic states has been referred to above, and that due to hereditary HPRT deficiency would reflect the erythrocyte enzyme defect as well as the liver defect; in the former condition the excessive output of orotate by the liver exceeds the capacity of the erythrocytes to metabolise the orotate, which is a function of its rate of PRPP synthesis. In hereditary erythrocyte pyrimidine 5'-nucleotidase deficiency (Harley et al., 1978; Valentine et al., 1974) the accumulation of erythrocyte nucleotides is a logical consequence of orotate uptake, since the K_m(apparent) for conversion of orotate to nucleotides in the erythrocyte is an order of magnitude lower than that for the conversion of uridine to nucleotides (Harley and Berman, 1984), which also explains why orotate is not normally detectable in the

plasma. The thymine-uraciluria found in patients with dihydropyrimidine dehydrogenase deficiency (Berger et al., 1984) does not resolve the question as to the predominant site of formation of uracil from uridine, but the report of massive excretion of uracil and thymine (but not uridine) in the urine of a child with medulloblastoma (Berglund et al., 1979) is consistent with the model as illustrated.

Understanding of the role played by the erythrocyte in the transport and metabolism of pyrimidines should also aid in the design and application of pyrimidine analogues in the treatment of malignant conditions and draw more attention to their potential use in the treatment of disorders and infections of the erythrocytes themselves.

CHAPTER 4

REPERFUSION INJURY IN RAT KIDNEYS

4.1 INTRODUCTION

There is an increasing body of evidence that reactive oxygen species are responsible for the tissue injury produced by reperfusion of ischaemic tissues with oxygenated blood. This has been referred to as reperfusion injury (Hearse, 1977; McCord, 1985; Braunwald and Kloner, 1985; Shlafer et al., 1982a and b; Granger et al. 1981; Flick et al., 1981; Manson et al., 1983; Parks et al., 1982; Grogard et al., 1982; Bulkley, 1983; Del Maestro and Arfors, 1986; Simpson and Lucchesi, 1987).

During ischaemia ATP is rapidly catabolised and accumulates as hypoxanthine and inorganic phosphate (Pi) (Arch and Newsholme, 1978; Saugstad, 1975; Miller et al., 1978; Harkness et al., 1980; Zimmer, 1984; Harkness et al., 1984; Giacomello and Salerno, 1984; Salerno and Giacomello, 1985). (In tissues that possess xanthine dehydrogenase, catabolism is able to proceed further to xanthine and uric acid, and in those animals that have uricase (not man), to allantoin). On account of rapid equilibration across the cell membrane (Lassen, 1967), the accumulation of hypoxanthine will be both intra- and extracellular. In ischaemic renal tissue hypoxanthine accumulation achieves levels of up to 300 times greater than normal (Osswald et al., 1977). At the same time the ATP deficit caused by anoxia results in the dissipation of trans-membrane electrochemical gradients which

in turn results in the flow of Ca^{2+} down its concentration gradient from the extracellular fluid into the cell. This increase in intracellular Ca^{2+} activates a proteolytic conversion of xanthine dehydrogenase (Type D) to xanthine oxidase (Type O) (Stirpe and Della Corte, 1969; Batelli et al., 1972; Waud and Rajagopalan, 1976), a form of the enzyme which transfers electrons from hypoxanthine and xanthine to oxygen instead of its normal substrate NAD^+, with the formation of superoxide (Roy and McCord, 1983; Slater, 1985; Chambers et al., 1985), (see Fig. 4.4).

The dramatic conversion of xanthine dehydrogenase to xanthine oxidase, demonstrated by Roy and McCord (1983) in support of this model for the generation of superoxide, occurred in rat intestinal segments that had been subjected to global ischaemia with almost total conversion of dehydrogenase (XD) to oxidase (XO) occurring within 1 minute. However, Engerson et al. (1987) in their studies on rats demonstrated that, in response to global ischaemia, tissue XD was converted to XO in all tissues with half-times of conversion at 37°C of 3.6, 6, 7 and 14 hours for the liver, kidney, heart and lung respectively. It is a matter of conjecture as to whether these significantly longer half times are in a time frame consistent with the ischaemia-reperfusion injury process. Furthermore, the activity of XO in brain (and some other tissues) is substantially lower than in other tissues (Del Maestro and Arfors, 1986, pp. 92 and 93), a tissue that is easily damaged by hypoxia (Siesjo, 1987). The inhomogeneity of distribution of the XD-XO enzyme system in endothelial cells and in a number of organs suggests that a

degree of variability may exist in the importance of this enzymatic free radical generation in the reperfusion of different organs after ischaemia. The absence of results, suggesting a role for scavenging enzymes (e.g. SOD) in cerebral ischaemia and reperfusion may reflect the low levels of these enzymes present in brain capillaries (Del Maestro and Arfors, 1986, epilogue).

Therapeutic approaches to tackle the problem of reperfusion injury have included the prevention of superoxide generation from hypoxanthine by the use of inhibitors such as allopurinol (Cunningham et al., 1974). A second approach is the removal of superoxide, as it is formed, by the use of free radical scavengers, such as superoxide dismutase (Del Maestro, 1980; Granger et al., 1981; Lefer et al., 1981; Shlafer et al., 1982a and b; Jolly et al., 1984).

A third approach would be to remove hypoxanthine from anoxic tissues prior to re-exposure to oxygen. In Chapter 2 the ability of human erythrocytes to metabolise hypoxanthine to IMP was demonstrated to be dependent on unphysiologically high phosphate concentrations. This has also been shown by Giacomello and Salerno (1979 and 1984); Hershko et al. (1967 and 1969); and Gutenson (1975). High phosphate concentrations have been demonstrated in ischaemic tissues (Garlick et al., 1979; Radda, 1983; Radda et al., 1983; and Arnold et al., 1984). The concentration of ATP in whole muscle has been measured at 5.5 mmol/kg (Harris et al., 1974). On complete degradation this could potentially

generate a local concentration of about 15 mM inorganic phosphate in the ischaemic tissue. Creatine phosphate would further contribute to the increase in phosphate concentration in cardiac and skeletal muscle; potentially another 35 mM. The inorganic phosphate concentrations generated in ischaemic tissue should therefore be sufficient to stimulate significant hypoxanthine uptake by erythrocytes either resident in capillaries in the anoxic tissue, or introduced on reperfusion of the anoxic tissue.

In this chapter, the development of a model of renal ischaemia in the rat is described. The ability of human erythrocytes, "primed" by preincubating in acid medium of high phosphate concentration and low oxygen tension, to take up hypoxanthine in a concentrative manner when perfused through ischaemic rat kidney is demonstrated. Attempts to demonstrate improved survival and renal function in rats whose ischaemic kidneys had been perfused with "primed" human erythrocytes prior to reperfusion were, however, unsuccessful.

It is further demonstrated that "unprimed" human erythrocytes, resident in ischaemic rat kidney for 3 hours, take up hypoxanthine and convert it to IMP. This suggests that erythrocytes could play a physiological role in the prevention of reperfusion injury.

4.2 MATERIALS AND METHODS

4.2.1 Washing of erythrocytes

Venous blood was sampled from healthy human volunteers into commercial, lithium heparin-containing vacuum tubes and centrifuged and washed as described in section (2.2.1).

4.2.2 Preparation of "unprimed" erythrocytes

Freshly washed human erythrocytes were resuspended to a haemotocrit of 0.33 in medium containing glucose (10 mM), HEPES (20 mM, and adjusted to pH 7.4 with NaOH) and NaCl to adjust to isotonicity. This suspension was designated "unprimed" erythrocytes.

4.2.3 Preparation of "primed" erythrocytes

Freshly washed human erythrocytes were suspended in medium, (packed cell volume to medium ratio approximately 1:4), containing glucose (10 mM), NaH_2PO_4 (20 mM, pH adjusted to 7.2 with NaOH), HEPES (20 mM, pH adjusted to 7.,2 with NaOH) and NaCl to adjust to isotonicity and incubated for 3 hours at 37°C with continuous gentle shaking. The suspension was then centrifuged for 5 minutes at 1700 x g and the packed erythrocytes resuspended in fresh medium of the same composition to a haematocrit of 0.33 and kept at 0-4°C till used later the same day.

4.2.4 Sampling of blood from rats

The rats were first anaesthetised in a glass bell jar containing ether and then blood was obtained from the retro-orbital plexus of veins by inserting the tip of a Pasteur pipette between the medial epicanthus and the medial border of the globe and allowing the blood to spontaneously flow into the pipette. No more than 1 ml of blood was sampled from any rat on a given day. The animals appeared to tolerate this procedure without any untoward effects; specifically, there was no adverse injury of local anatomy and there did not appear to be any visual impairment.

Immediately after sampling, the blood was inserted into glass tubes, allowed to clot and then centrifuged at approximately 3000 x g for 5 minutes. The separated serum was used for creatinine estimation.

4.2.5 Preparation of erythrocytes for HPLC

Erythrocyte suspensions were prepared for HPLC as described in section 3.2.1.

4.2.6 HPLC

High pressure liquid chromatography (HPLC) was carried out as described in section 2.2.5.

4.2.7 Creatinine determination

0.5 ml of serum was mixed with 0.5 ml H_2O, 0.5 ml 5% Sodium Tungstate and 0.5 ml 0.33 M H_2SO_4, and allowed to stand for ten minutes. The precipitate was then deposited by centrifugation and then 250 µl saturated Picric Acid and 250 µl 0.75 M NaOH added to 750 µl of the supernatant and the mixture allowed to stand for 20-45 minutes before reading the absorbance at 520 nm. A 100 µM creatinine standard was used and H_2O used as a blank.

4.2.8 The model and surgical procedure

Adult male Long Evans (L.E.) rats weighing between 250 and 350 g were anaesthetised by placing them in a glass bell-jar containing ether-soaked cotton wool and then given intramuscular Ketamine Hydrochloride (Ketalar) in a dose of 1 mg per 100 g body weight. The animals were then taped by their ankles, ventral surface up, to a cork-surfaced operating board and anaesthesia maintained using ether in a cottonwool-containing nose cone, and intermittent "topping up" doses of 0.1 - 0.2 mg Ketalar per 100 g body weight. Survival is improved if minimal ether anaesthetia is used and smaller, more frequent doses of Ketalar administered as required rather than a single maximum bolus dose.

After shaving the fur from the abdominal wall and cleansing the skin with Hibitane in alcohol the abdomen was entered via a longitudinal incision from the xiphisternum and extending

sufficiently inferior to allow adequate access for the isolation of the kidneys. Appropriately bent paper clips attached to rubber bands anchored to the operating board served as retractors bilaterally.

After reflecting the liver superiorly out of the abdominal cavity and maintaining it there with a saline soaked gauze swab the intestines were reflected extracorporeally to the animal's left to allow access to the right kidney which was then removed after dissecting it free from the posterior abdominal wall and placing a silk ligature around the renal vessels and ureter.

To gain access to the left kidney, the intestines were reflected extracorporeally to the animal's right and covered with a saline-soaked gauze swab.

Microsurgical instruments and an operating microscope (Nikon) with a capacity for 4-40 times magnification were used for the microsurgery described below.

In this model the left kidney has a fairly long vascular pedicle, usually comprising a large renal vein and a smaller renal artery, which, proximally, lies posterior to the vein but on its course to the renal hilum lies superiorly and then antero-superiorly to the vein at the hilum. The adrenal artery arises from the superior aspect and approximately half-way along the renal artery. In four percent of the 200

animals dissected there was some variation in this pattern. In some, the renal artery was double, with origins from the aorta above and below the renal vein and in a smaller proportion there was an anomalous blood supply to the kidney, one from a branch of the adrenal artery to the upper pole of the kidney and in another animal there was an early division of the renal artery after its takeoff from the aorta. Such anomalies made cannulation and control of the blood supply difficult and these animals were therefore excluded on identification.

After cauterisation of the adrenal artery, the (left) renal artery and vein were dissected free from surrounding tissue so as to allow the placement, but not tying, of silk ligatures proximally and distally on each vessel. The proximal ligature serves to occlude blood flow and the function of the distal ligature, when required, is to retain and prevent leakage past an intravascular cannula. To ensure complete ischaemia, the peritoneum over the periphery of the kidney was incised and the kidney then dissected free from its retroperitoneal bed, and the ureter with its colateral vessels clamped.

Prior to occluding any vessels, the rat was heparinized by injecting 0.1 units of heparin per gram body weight via the dorsal vein of the penis using a 26 gauge needle. The solution of heparin diluted in saline containied 50 units per ml; each rat therefore required approximately 30 units of heparin and this was given in a volume of 0.6 ml.

Following heparinisation, the ureteric clamp was placed and the proximal arterial ligature tightened. Adequate ischaemia was evidenced by immediate blanching of the kidney. If this was apparent, the time was noted and the vein similarly ligated. The ischaemic interval was the time elapsed between renal artery ligation and the kidney reperfusion that occurred after this (proximal) renal artery ligature was cut free.

Having ligated the vessels and rendered the kidney ischaemic, where indicated, the next step was to cannulate the renal vessels. The renal artery between the tight proximal ligature and the loose distal one was cleaned of loose connective tissue and small branches cauterized. A ligature tied around the base of the overlying adrenal vein as it enters the renal vein made a useful handhold for retracting the renal vein, thus improving access to the renal artery. After exposure and cleaning of the renal artery a small arteriotomy was performed using microscissors and the vessel cannulated with a 24 gauge "Bardi-cath" catheter (C.R. Bard International Ltd., Sunderland, England; P. No. 2012), previously primed with the appropriate solution, and secured by tying the distal ligature. The catheter was used over and again by keeping it clean, using Hibitane and water, but not sterile. It could be kept soft and pliable by occasionally rubbing it with glycerine. The vein was cannulated in a similar manner using a 22 gauge "Jelco"

intravenous catheter (Criticon, Tampa, USA; order number 4053). The perfusion conditions are defined for each experiment. The flow rate was 0.5 ml/min unless specified otherwise.

4.2.9 Perfusion apparatus

A peristaltic, non-occlusive, pump (LKB, Bromma, USA) was attached to the arterial cannula using fine bore siliconized tubing to carry the specified perfusate from a reservoir immersed in a 37°C water bath to the 24 gauge arterial cannula. When required, the perfusate eluting from the kidney via the 22 gauge renal vein cannula was collected. Appropriately cut Biofusion anaesthetic extension sets (Biofusion (Pty) Ltd, Johannesburg, South Africa; E x 90) proved useful in making some of the necessary connections. When indicated, a three-way stop-cock interposed between the lines from two "feeding" solutions (saline and blood) in the 37°C water-bath and the peristaltic pump simplified the changing from one perfusing solution to the other.

On completion of the perfusion the cannulae were removed in reverse order and the vessels repaired. The vein was repaired using a continuous 8-0 nylon suture and the artery with interrupted 10-0 nylon sutures. After the repairs, first the venous and then the arterial ligatures were removed to re-establish normal renal blood-flow. The kidney returned to its normal colour within three minutes if the repair was adequate.

4.2.10 Deoxygenation of solutions

Solutions that were used to perfuse kidneys after a period of ischaemia were deoxygenated to a pO_2 < 3.0 kPa by slowly bubbling helium gas through the solutions (in their capped reservoirs suspended in the 37°C water-bath) for at least half an hour prior to use.

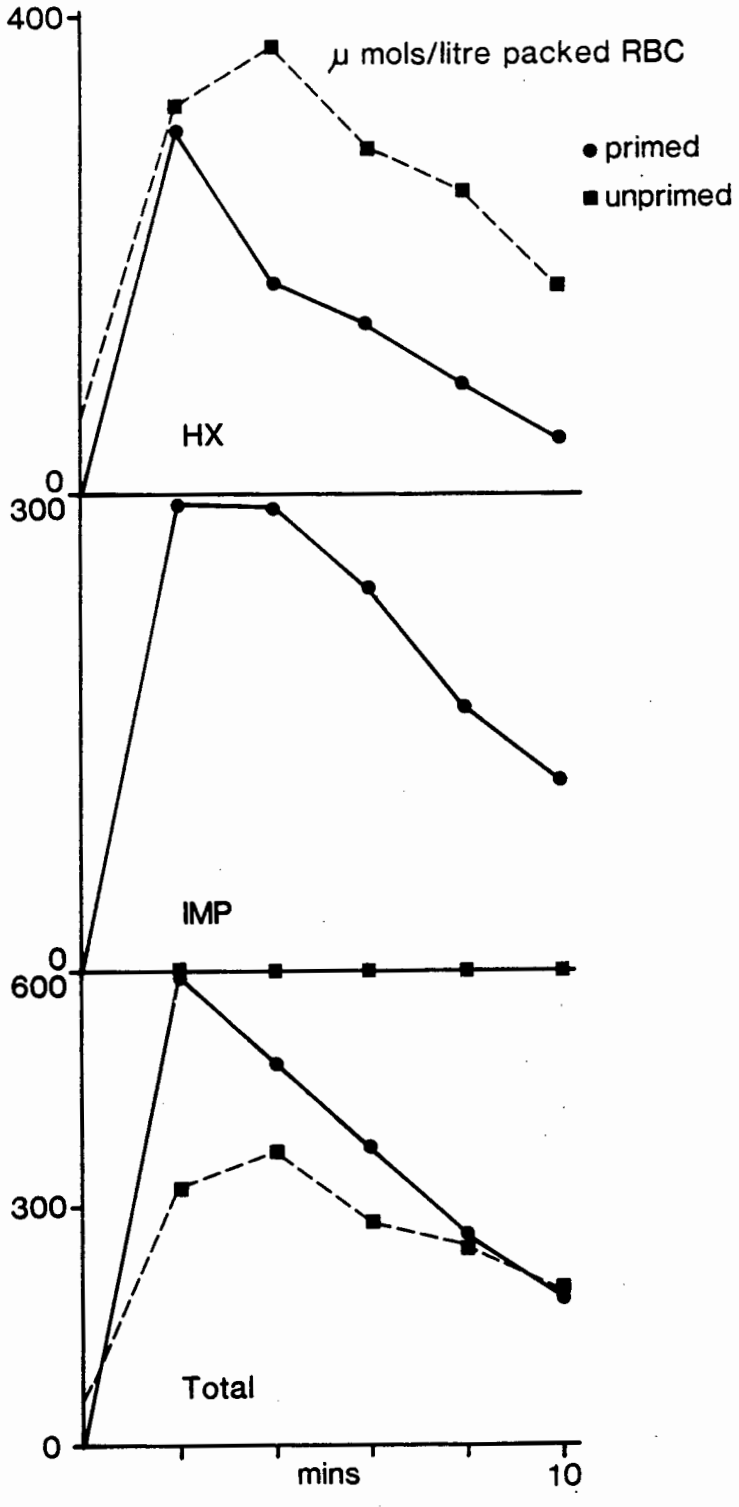
4.3 RESULTS

4.3.1 The ability of "primed" erythrocytes to form IMP from hypoxanthine in ischaemic rat kidney

To investigate the ability of primed erythrocytes to take up hypoxanthine (Hx) from ischaemic rat kidney and convert it to inosine 5'-monophosphate (IMP), ischaemic rat kidneys were separately perfused with either "primed" or "unprimed" erythrocytes. The effluent erythrocytes were analysed for their hypoxanthine and IMP content using reverse phase and anion exchange HPLC. The results are shown in Figure 4.1 and demonstrate that "primed" erythrocytes are more efficient at clearing the ischaemic kidney of hypoxanthine than "unprimed" erythrocytes. This can be deduced from the fact that tissue and erythrocyte hypoxanthine levels equilibrate within a matter of seconds at 37°C (Muller and Falkner, 1976); the erythrocyte hypoxanthine levels thus closely reflect the tissue hypoxanthine levels. The results demonstrate that the primed erythrocytes are able to do this by converting hypoxanthine to IMP, thus taking hypoxanthine

Figure 4.1. The ability of "primed" erythrocytes to form IMP from hypoxanthine in ischaemic rat kidney. Isolated rat kidneys were subjected to warm renal ischaemia for 1 hour and then separately perfused with either "primed" or "unprimed" human erythrocytes. The effluent erythrocytes were analysed for their hypoxanthine and IMP contents using reverse phase and anion exchange HPLC. Hx, hypoxanthine; IMP, inosine 5'-monophosphate; RBC, erythrocytes; Total, total oxypurine content (i.e., Hx + IMP). ●——● , "primed" erythrocytes; ■——■ , "unprimed" erythrocytes.

FIG. 4.1


400
μ mols/litre packed RBC
● primed
■ unprimed
HX
0
300
IMP
0
600
300
Total
0
mins
10

up in a concentrative manner. In contrast, no IMP was detected in the unprimed erythrocytes. It can be seen that because of this concentrative uptake of hypoxanthine, the total oxypurine (hypoxanthine + IMP) content of the "primed" erythrocytes at two minutes is double that of the "unprimed" erythrocytes. This difference decreases with the passage of time as the hypoxanthine level in the kidney perfused with "primed" cells decreases more rapidly, and at 10 minutes hypoxanthine levels are less than one-third those found in the kidney perfused with "unprimed" cells. Figure 4.2 shows the anion exchange HPLC profiles of erythrocytes eluting during the third minute of perfusion. The presence of the IMP peak in the "primed" erythrocytes, and its absence in the "unprimed" erythrocytes is demonstrated. The identity of the IMP peak was confirmed by spiking. The identity of the hypoxanthine peak was confirmed by spiking and by the fact that the peak was eliminated by the addition of xanthine oxidase (Boehringer) to a sample prior to HPLC analysis.

4.3.1 Ability of "primed" human erythrocytes to remove hypoxanthine from ischaemic rat kidney

To demonstrate that "primed" erythrocytes are more efficient than "unprimed" erythrocytes at removing hypoxanthine from ischaemic tissue, the renal artery and vein of kidneys that had been removed from adult male Long Evans rats were cannulated and incubated in a humidified environment at 37°C for 1 hour. The kidneys were then perfused at a rate of ½ ml per minute with either "primed" or "unprimed" erythrocytes

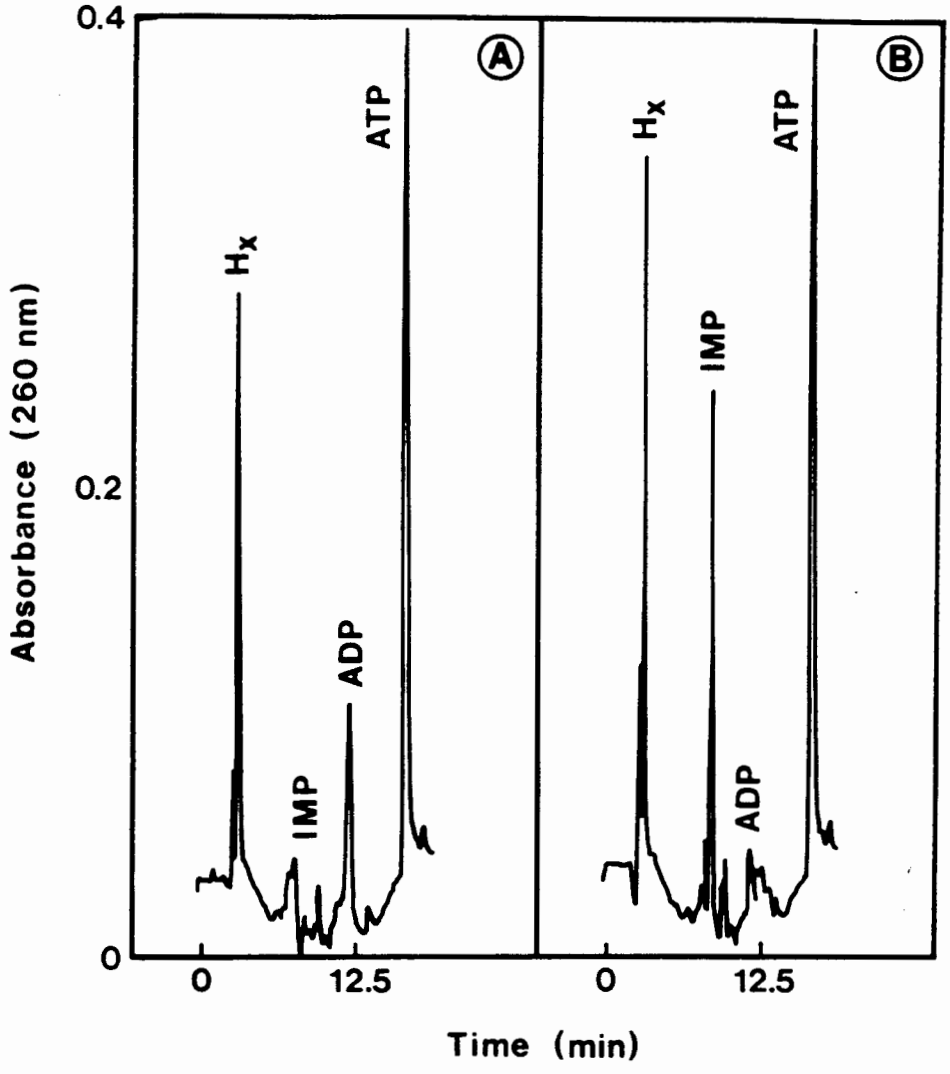


Figure 4.2. HPLC profiles of acid soluble material in "unprimed", A, and "primed", B, human erythrocytes after perfusion through ischaemic rat kidney. Neutralised PCA extracts of "primed" and "unprimed" human erythrocytes eluting from ischaemic rat kidney during the third minute of perfusion in the experiment described in Figure 4.1 were subjected to anion exchange HPLC to quantitate their inosine 5'-monophosphate (IMP) content. Chromatographic conditions are described in section 2.2.5.

Hx, hypoxanthine.

for 10 minutes. The kidneys were then snap frozen in liquid nitrogen, freeze dried and then analysed for their hypoxanthine content using HPLC. The contralateral kidney, which was also incubated for 70 minutes at 37°C but not perfused, was used as a control in each perfusion experiment. The results, which are given in Table 4.1, show that "primed" erythrocytes are more efficient at removing hypoxanthine from ischaemic rat kidney than "unprimed" erythrocytes.

4.3.3 The ability of "unprimed" human erythrocytes resident in ischaemic rat kidney to take up and convert hypoxanthine to IMP

PRPP formation in erythrocytes is partly dependent upon the presence of unphysiologically high levels of inorganic phosphate (Pi) (Hershko et al., 1969; Giacomello and Salerno, 1979). It is known that Pi levels in ischaemic tissue rise significantly, possibly as high as 20-25 millimolar (Garlick et al., 1979; Osswald et al., 1977; Radda, 1983; Radda et al., 1983; Sorlie et al., 1982). To determine whether "unprimed" human erythrocytes resident in ischaemic tissue are exposed to sufficiently high levels of Pi to stimulate the formation of PRPP and thus the uptake and conversion of hypoxanthine to IMP, human erythrocytes suspended in HEPES (20 mM; pH 7.4), glucose (10 mM) and adjusted to isotonicity with NaCl (but no Pi) were incubated in duplicate in ischaemic rat kidney for 3 hours at 37°C. The erythrocytes were then flushed out, separated from the medium by centrifuging through dibutylphthalate and then

TABLE 4.1

Hypoxanthine content of ischaemic rat kidney after the passage of either "primed" or "unprimed" human erythrocytes.

		μg Hx/g kidney (dry wt)		
		Control	Test	Difference
"Unprimed" erythrocytes	1	1269	1050	219
	2	580	340	240
	3	429	103	326
"Primed" erythrocytes	4	810	271	539
	5	1152	325	877
	6	1303	475	828

Hx, hypoxanthine.

analysed for their IMP and hypoxanthine contents using HPLC. Control erythrocytes, suspended in the same medium but not perfused into a kidney, were incubated *in vitro* for the same time period, also at 37°C. The results are given in Table 4.2 and show that IMP accumulated in the "resident" erythrocytes to a mean level of 68 micromoles per litre of packed erythrocytes. Predictably, no hypoxanthine was detectable in the control erythrocytes. The mean hypoxanthine level in the "resident" erythrocytes was 315 micromoles per litre of packed erythrocytes, a figure in agreement with the levels found in ischaemic rat kidneys by Osswald *et al*. (1977). ATP levels in both control and test erythrocytes were in the physiological range.

4.3.4 Effect of storage on the ability of "primed" erythrocytes to convert hypoxanthine to IMP

To determine the effect of storage on the ability of "primed" erythrocytes to convert hypoxanthine to IMP a batch of "primed" erythrocytes was prepared as described in section 4.2.3, stored at 4°C, and their ability to convert hypoxanthine to IMP analysed at weekly intervals by incubating at 37°C for 10 minutes in medium containing HEPES (20 mM, pH adjusted to pH 7.2 with NaOH); hypoxanthine, 1 mM; and adjusted to isotonicity with NaCl. The erythrocytes were suspended in the medium to a haemotocrit of 0.25. The cells were separated from the medium and prepared for HPLC as described in section 2.2.1. Anion exchange HPLC was carried out as described in section 2.2.5. The results are given in

TABLE 4.2

Purine content of human erythrocytes resident in ischaemic rat kidney

	Hx micromoles/l packed rbc's	IMP micromoles/l packed rbc's	ATP mmoles/l packed rbc's
Control	nil detected	nil detected	1.0
Test 1	310	64	1.5
Test 2	320	71	1.3
Mean of 1 & 2	315	68	1.4

Hx, hypoxanthine; IMP, inosine monophosphate.

Table 4.3 and show that there is a decrease from 320 micromoles per litre packed erythrocytes on day 0 to 120 micromoles per litre packed erythrocytes on day 14.

4.3.5 Survival statistics in heminephrectomised rats subjected to warm renal ischaemia (not cannulated)

To establish survival statistics four cohorts of ten rats were heminephrectomised and the remaining (uncannulated) kidney rendered ischaemic for varying lengths of time (specified), each cohort being subjected to different periods of ischaemia. Blood samples were taken on the days indicated for plasma creatinine determination and the survival of the animals recorded. The results are given in Table 4.4 and reveal that after 1, 1.5, 2.25 and 3 hours ischaemia the mortality rates at day 10 were 0, 10, 70 and 90%, respectively. Typically, plasma creatinine rose to maximum levels on day 2, whereupon the rats either died or the plasma creatinine dropped back to normal levels by day 8.

4.3.6 Development of renal vascular cannulation and perfusion technique

To develop a renal vascular cannulation and perfusion technique, 40 Long Evans rats were subjected to left nephrectomy and a variety of methods of isolating and perfusing the remaining right kidney. The technique which produced the most favourable survival statistics was evolved and is described in section 4.2.8.

TABLE 4.3

Effect of storage on "primed" erythrocytes

Human erythrocytes were prepared, stored and analysed in duplicate for their ability to convert hypoxanthine to IMP. The mean values are shown and were found to vary from their duplicates by less than 10%.

Day	IMP micromoles/l packed rbc's
0	320
7	174
14	120

IMP, inosine monophosphate

TABLE 4.4

Survival statistics in heminephrectomised rats subjected to warm renal ischaemia (not cannulated)

Four cohorts of ten heminephrectomised rats were each subjected to different periods of renal ischaemia and their subsequent survival and plasma creatinine levels followed.

Renal ischaemic time (hours)	Day	Mean plasma creatinine in mg/100 ml (1 SD)	Survival
1.0	0	1.16 (0.41)	
	2	1.41 (0.39)	
	4	1.22 (0.08)	100%
	6	1.28 (0.24)	
	8	1.41 (0.23)	
	10	1.24 (0.19)	
1.5	0	1.36 (0.23)	
	2	2.87 (1.27)	
	4	1.59 (0.28)	90%
	6	1.16 (0.24)	
	8	1.09 (0.15)	
2.25	0	0.81 (0.28)	
	2	7.50 (2.94)	
	4	4.72 (3.89)	70%
	6	2.20 (1.79)	
	8	1.24 (0.14)	
3.0	0	1.27 (0.17)	
	2	9.8 (3.65)	10%
	4	2.17 (3.65)	
	6	1.35 (3.65)	

SD, standard deviation.

Concomitantly, attempts were made to reproduce the results shown in Table 4.4, but with the left renal artery cannulated and subsequently repaired. Without exception, rats subjected to more than 90 minutes of warm renal ischaemia, with associated renal artery cannulation and repair, died within 48 hours. Thus, the protocol of 1 hour total warm renal ischaemia, with a 10 minute period of perfusion included, was evolved. The perfusion period of 10 minutes was selected somewhat arbitrarily as this allowed time for cannulation, perfusion and vascular repair, all within the hour's ischaemic time. As the operator became more skilled, the vascular repair took less time and the perfusion period could thus be extended. For the purposes of this study, however, the perfusion period was kept at 10 minutes.

It was found that the cannulation and repair of the renal vasculature significantly reduced the warm renal ischaemia which a heminephrectomised rat can tolerate. From a mortality rate of 10% after 1.5 hours warm renal ischaemia with no breaching of vessel walls, the mortality rate rose to 70% for the same period of ischaemia, but with associated cannulation and repair.

4.3.7 Survival in rats subjected to warm renal ischaemia and subsequent perfusion with "primed" erythrocytes

To determine if the perfusion of kidneys of rats subjected to warm renal ischaemia with "primed" erythrocytes could improve their survival, four cohorts of twenty adult male Long Evans

rats were established. One cohort was the experimental group and these animals were subjected to perfusion with freshly "primed" erythrocytes (haematocrit, 0.33). Three control groups formed the other cohorts and comprised the following:

(a) Technique control: animals were subjected to renal artery and vein cannulation and repair but were not perfused.

(b "Unprimed" erythrocyte control: the cannulated kidneys were perfused for 10 minutes with fresh, saline washed, "unprimed" human erythrocytes suspended in normal saline to a haematocrit of 0.33.

(c) Perfusion control: renal arteries and veins were cannulated and repaired but the kidneys were perfused with warm saline only.

Apart from the perfusion differences between the four cohorts all animals were treated similarly. The operative and perfusion techniques are described in sections 4.2.8 and 4.2.9, respectively. Plasma creatinine levels were determined by the method described in section 4.2.7. The survival rates of the four cohorts at day 8 are given in Table 4.5. A one-way analysis of variance was used to compare the four cohorts, and no significant differences in survival could be found. The mean plasma creatinine levels are given in Table 4.6 and in graphical form in Figure 4.3. In concordance with the survival rates, no significant

TABLE 4.5

Survival rates post warm renal ischaemia and cannulation, with and without perfusion.

Cohort	Number of rats surviving					
	Day: 0	1	2	4	6	8
No perfusion	19	18	11	9	8	7
"Unprimed" erythrocytes	18	10	6	7	6	6
"Primed" erythrocytes	19	8	8	7	6	6
Saline	21	15	8	6	6	6

TABLE 4.6

Mean plasma creatinine levels post warm renal ischaemia and cannulation, with and without perfusion

These results are presented in graphical form in Figure 4.3.

Cohort	Plasma creatinine in mg % (1 Std. Dev.)					
	Day: 0	1	2	4	6	8
No Perfusion (n = 19)	1.02 (0.37)	3.4 (1.02)	3.9 (1.9)	2.14 (1.2)	2.18 (1.3)	1.54 (0.68)
"Unprimed" erythrocytes (n = 18)	1.16 (0.73)	4.46 (1.0)	1.95 (0.54)	1.81 (0.61)	1.76 (0.9)	1.3 (0.47)
"Primed" erythrocytes (n = 19)	0.88 (0.33)	4.2 (0.66)	4.32 (2.09)	2.29 (1.5)	1.5 (1.12)	1.18 (0.46)
Saline (n = 21)	1.15 (0.46)	4.78 (0.92)	4.8 (1.4)	3.9 (1.6)	2.98 (1.67)	1.8 (0.53)

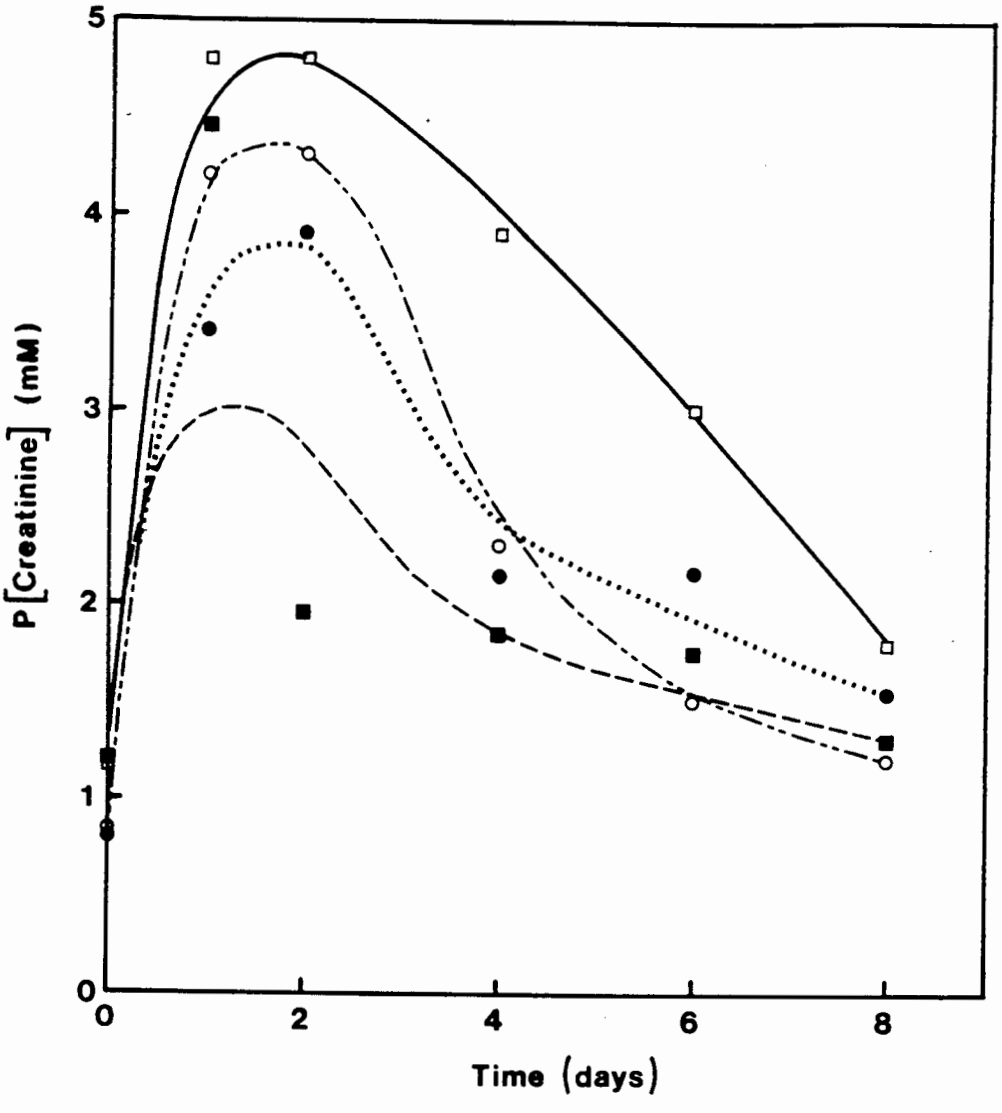


Figure 4.3. Mean plasma creatinine levels post warm renal ischaemia and cannulation, with and without perfusion. Four cohorts of approximately 20 rats were subjected to warm renal ischaemia and cannulation, with and without perfusion as described in section 4.3.7. The graphs in this figure are plotted from the results presented in Table 4.6 where the number of rats in each cohort and the standard deviations of the mean values are given. ■---■, no perfusion; ○---○, perfused with "unprimed" erythrocytes; ●·····●, perfused with "primed" erythrocytes; □——□, perfused with saline.

differences are seen between the creatinine levels in the four cohorts.

4.4 DISCUSSION

Superoxide radical-induced cytotoxicity appears to depend largely on the subsequent production of a highly reactive species, the hydroxyl radical (or its redox equivalent) catalyzed by iron, which is probably in the form of an organic iron complex (Wilson, 1984; Halliwell and Gutteridge, 1984), (see Fig. 4.4). Upon reperfusion of previously ischaemic tissue, reactive oxygen species are implicated as the cause of increased permeability of cell membranes of endothelium and epithelium. Increased permeability in cell membranes of tubular epithelial cells or capillary endothelial cells could lead to disruption of vital transport functions. Membrane damage by reactive oxygen species could occur at the plasmalemma with secondary release of other mediators such as lipid endoperoxides, arachidonic acid, and hydroperoxides. Either these mediators or the initiating species could induce endothelial cell contraction terminating in a macromolecular leak (Del Maestro et al., 1980). The hydroxyl free radical has been shown to degrade hyaluronic acid (McCord, 1974), which is one of the principal constituents of the interstitial matrix and the capillary basement membrane (Altura, 1980). Oxygen free radical-induced damage to the capillary basement membrane could exacerbate the microvascular leak already established as a result of endothelial cell damage.

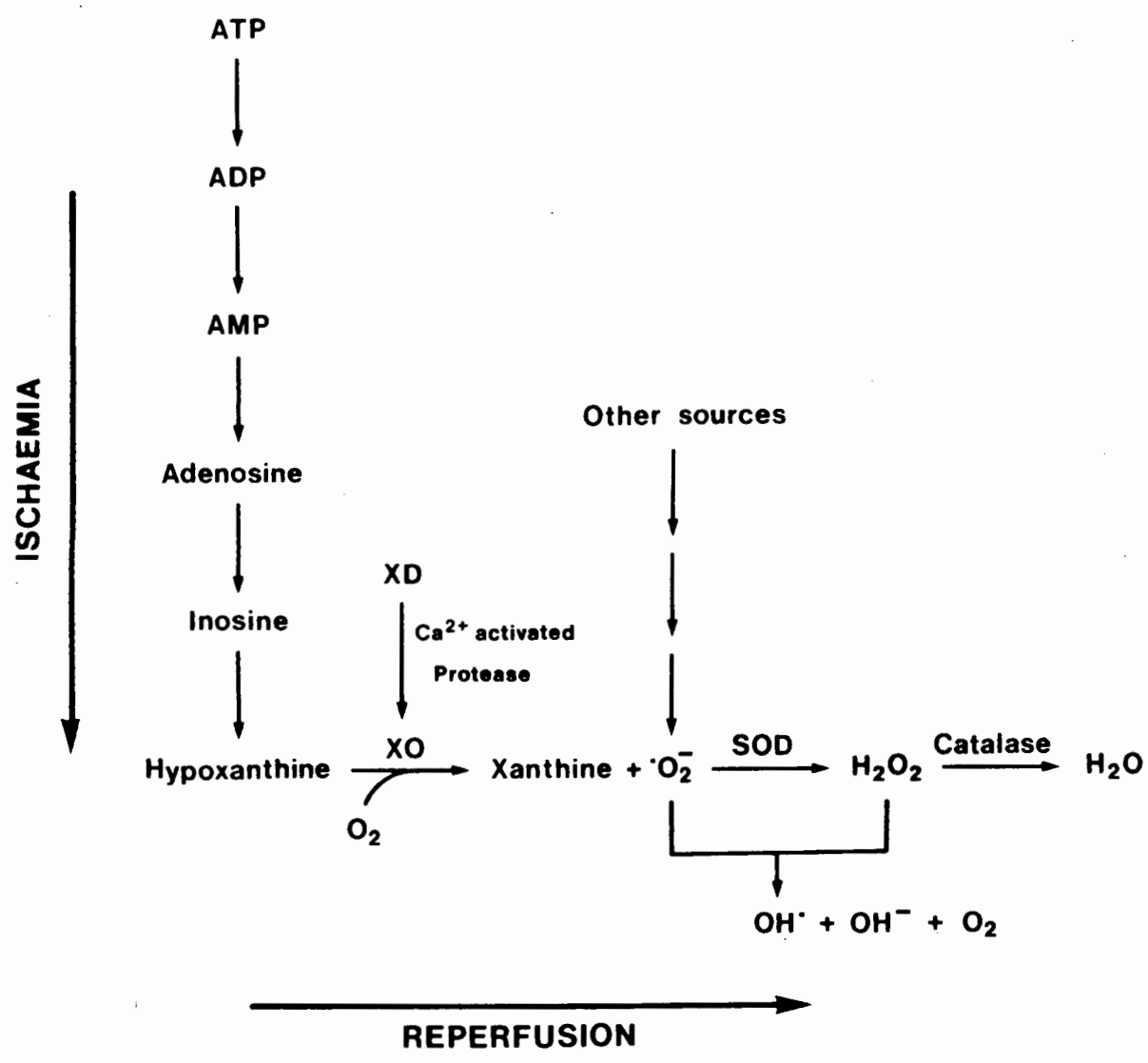


Figure 4.4. Scheme depicting the proposed mechanism for the production of superoxide anion free radical ($O_2^{\cdot-}$) and other reactive oxygen species after transient ischaemia followed by reperfusion with oxygenated blood. Superoxide dismutase (SOD) catalytically removes $O_2^{\cdot-}$ generated from all possible sources (modified from McCord, 1985). XD, xanthine dehydrogenase; XO, xanthine oxidase; SOD, superoxide dismutase; $O_2^{\cdot-}$, superoxide; $OH^\cdot$, hydroxyl radical.

The findings of Baker et al. (1985) support the proposal that $O_2^{\bullet-}$ plays a critical role in the structural and functional damage to kidneys that results from reoxygenation of ischaemic tissue and further that the oxygen radicals are in part derived from the action of xanthine oxidase.

The studies in this thesis demonstrate that human erythrocytes, "primed" by preincubating in medium of low pH and high inorganic phosphate concentration to increase their (intracellular) PRPP levels, are able to take up hypoxanthine from ischaemic rat kidney in a concentrative manner by converting it to IMP, whereas "unprimed" erythrocytes are not able to do so. It is also demonstrated that these "primed" erythrocytes are quantitatively more efficient at removing hypoxanthine that has accumulated in ischaemic rat kidney. However, after development of a rat model of renal ischaemia and a technique of vascular cannulation and renal perfusion, no improved survival or renal function could be demonstrated in rats whose ischaemic kidneys were perfused with "primed" human erythrocytes over those rats whose ischaemic kidneys were perfused with "unprimed" cells. Whilst this result does not support the role of oxygen free radical-induced damage in kidneys subjected to warm ischaemia and reperfusion, as did the work of Baker et al. (1985), it does not disprove it, as, whilst the "primed" cells were more efficient at hypoxanthine removal from the ischaemic tissue there was still some hypoxanthine remaining in the kidneys at

the time of reperfusion, and this could have been sufficient to lead to an initiation of the free radical chain reaction that could result in the destruction of cells (Esterbauer et al., 1982).

Furthermore, a longer ischaemic time (approximately 2½ hours) was required to produce 50% survival in the (Long Evans) rats used in the experiments described in this thesis, whereas only 45 minutes ischaemic time was required in the experiments of Baker et al. (1985) where male LBNF 1 rats were used. There is no immediately apparent explanation for this difference; certainly, the renal ischaemia in the experiments described here was complete. Perhaps the defence mechanisms against reperfusion injury (e.g. superoxide dismutase activity) of the Long Evans rats used here are more effective than those of the rats used by Baker et al. The relevance of the longer ischaemic time used here to produce injury, is that whilst superoxide radicals might act as primary mediators of ischaemic tissue injury, with more severe or prolonged periods of ischaemia, other factors such as ATP depletion and proteolysis may play a more important role in the chemical and structural alteration of tissue (Baker et al., 1985). These other factors could then negate the protective effects offered by lowering tissue hypoxanthine in ischaemic tissue prior to reperfusion with oxygen rich blood.

The effect of storage on the capacity of "primed" human erythrocytes to convert hypoxanthine to IMP has here been determined, indicating that this capacity is approximately halved in 7 to 10 days of storage at 4°C. This has time-saving implications in conducting experiments in the future; fresh batches of "primed" erythrocytes need not be prepared every day.

Also demonstrated here, is that unprimed human erythrocytes left resident in ischaemic rat kidney for 3 hours, are able to take up accumulated hypoxanthine and convert it to IMP. One can conclude then, that to account for the conversion of hypoxanthine to IMP, PRPP has been generated in these erythrocytes, indicating that sufficient inorganic phosphate and hydrogen ions have accumulated in the ischaemic tissue to promote the formation of PRPP (see Introduction of this chapter). This has possible pathophysiologic implications. Perhaps, during a temporary cessation in blood flow to an organ (such as may occur in the kidneys and intestines of the injured mammal in hypovalaemic shock) the erythrocytes trapped in the ischaemic tissue are able to generate sufficient PRPP to remove enough accumulating hypoxanthine from the tissue to reduce reperfusion injury should blood flow be re-established.

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